Self-fertilization, Larval Dispersal, and Population Structure in the Marine Bryozoan Bugula stolonifera

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Abstract

Although the process by which fertilization occurs in bryozoans is well described, the ability to self-fertilize and the subsequent ecological consequences are poorly understood. Culturing experiments were conducted examining the effects of selfing on offspring survival and reproduction in the simultaneous hermaphrodite *Bugula stolonifera* collected from Eel Pond, Woods Hole, MA. Results from these experiments document significant decreases in survival and fecundity of selfed offspring, compared to outcrossed controls, suggesting that these animals are not routinely self-fertilizing in Eel Pond. How these arborescent colonies minimize selfing remains unclear, but it is hypothesized that conspecific aggregations could serve to minimize the chances that a colony utilizes its own sperm for fertilization. The genetic composition of these aggregations was investigated using a newly developed microsatellite library. As larvae routinely metamorphose on conspecific colonies, the possibility that larvae select or avoid their maternal colony was also investigated. Analyses of genetic structure document homogeneity throughout these aggregations on extremely small spatial scales, suggesting high amounts of larval dispersal within aggregations. When combined with results from parentage-exclusion and kinship analyses, these results indicate that a

colony's nearest neighbors are not composed of siblings, potentially minimizing inbreeding.

Molecular analyses were then used to determine if the high larval dispersal within aggregations resulted in high mixing between aggregations. Sites within Eel Pond separated by 100-300 m were routinely sampled from 2009 to 2011, and analyses were conducted to investigate potential inter- and intra-annual genotypic differentiation within and between aggregations. Results document that although low levels of mixing could result in increased homogeneity between some aggregations, barriers to genetic exchange prevent mixing between most sites. Further, inter-annual comparisons within sites document that significant differentiation can occur between reproductive seasons. Hence, any potential homogeneity achieved between sites during one reproductive season will likely be lost by the beginning of the next reproductive season. Additionally, while sampling in Eel Pond in 2010, I document the first occurrence from the western Atlantic Ocean of another aggregating arborescent bryozoan, *Tricellaria inopinata*. The growth and reproductive biology of these animals was monitored throughout 2011; results suggest that this introduction is likely to persist.

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For Meghan, Aiden, and Baby J

Chapter 1

Introduction

Bryozoans are simultaneous hermaphrodites that have long been thought to be capable of self-fertilization (see Silén 1966 and references therein). The process by which fertilization occurs in bryozoans has been well described (*e.g.*, Temkin 1994; 1996), but the occurrence of selfing in these animals remains unclear. For instance, Gooch and Schopf (1970, 1971) examined protein polymorphism in natural populations of *Bugula stolonifera* Ryland, 1960 and *Schizoporella unicornis*[1] (Johnston in Wood, 1844) from Woods Hole, MA and in *S. errata*[1] (Waters, 1878) from various locales between Cape Cod, MA and Beaufort, NC and found that allele frequencies for all species did not deviate from Hardy-Weinberg equilibrium (HWE), thus providing evidence that these colonies were routinely outcrossing. Yund and McCartney (1994) and McCartney (1997) used allozyme markers of *Celleporella hyalina* (Linnaeus, 1767) colonies collected from Walpole, ME to establish paternity in brooded embryos, and documented that some embryonic genotypes were consistent with selfing. In contrast, Hoare et al. (1999) used microsatellite markers to demonstrate that populations of *C. hyalina* located in Wales and England were in HWE. Finally, Watts and Thorpe (2006) used allozyme electrophoresis to demonstrate that most loci from four species of bryozoans (two brooders, two non-brooders) collected from coasts throughout Great Britain did not deviate significantly from predicted HWE. Although important in understanding the reproductive ecology of bryozoans, these studies only indirectly examine selfing in bryozoans, and cannot fully demonstrate if it occurs in these animals.

Maturo (1991) conducted the first direct examination of selfing in bryozoans, and found that colonies from six brooding gymnolaemate species reared in isolation were able

[1] The *Schizoporella unicornis* and *S. errata* colonies used by Gooch and Schopf are believed to have been incorrectly identified and should have been documented as *Schizoporella variabilis* Leidy, 1855 (personal communication J. E. Winston)

to release larvae. Interestingly, these six species were composed of both ctenostomate and cheilostomate bryozoans, suggesting that the ability to self is widespread in this group. What remains unknown from this study, however, is if these larvae were able to initiate and complete metamorphosis, and if there were any deleterious effects associated with selfing. Working with a species of bryozoan that utilizes a planktonic larval stage, Temkin (1991) found that isolated colonies of *Membranipora membranacea* (Linnaeus, 1767) from Friday Harbor, WA were able to release oocytes possessing sperm DNA, which subsequently developed into larvae. Although he found no significant difference in the percentage of these oocytes that developed into larvae between isolated and field-collected colonies, he was unable to culture these larvae to metamorphic competence due to the extremely long larval duration. Hunter and Hughes (1993) and Hoare and Hughes (2001) cultured offspring from *C. hyalina* collected from the Menai Straight, Wales, in isolation and found that larvae released from isolated colonies did not initiate metamorphosis. Interestingly, isolated colonies of *C. hyalina* collected from populations other than those used previously were able to self with no measureable decrease in offspring survival (Hughes et al. 2002; 2009). The effects of selfing on offspring reproductive fitness, however, have not been investigated for any species of bryozoan.

The lack of a clear understanding of selfing in bryozoans provided the impetus for this dissertation. The remainder of this chapter provides background information for this thesis, including a general description of bryozoan structure, phylogenetic history, reproductive processes, and larval dispersal capability. Chapter 2, published as Johnson (2010), details experiments examining the effects of self-fertilization on survival and offspring reproductive fitness in the marine bryozoan *Bugula stolonifera*. Chapter 3,

published as Johnson and Woollacott (2010), investigates the genetic make-up within conspecific aggregations of *B. stolonifera* in Eel Pond as a potential means to minimize selfing in this species. Chapter 4, Johnson and Woollacott (In press), examines the genetic structure of *B. stolonifera* aggregations in Eel Pond to determine the extent to which genetic exchange occurs between these aggregations. Finally, Chapter 5, Johnson et al. (In press), documents the first occurrence the widely distributed arborescent bryozoan *Tricellaria inopinata* d'Hondt and Occhipinti-Ambrogi, 1985 from the western Atlantic Ocean.

Bryozoan description and structure

Bryozoans are colonial invertebrates that can be conspicuous components of aquatic communities, often found attached to natural and artificial submerged surfaces. The majority of species are sessile, although free-living (*e.g.*, Cook 1965) and pelagic (*e.g.*, Peck et al.1995) species have been described. The life cycle of bryozoans is similar to that of other indirect developers; sexual reproduction results in the formation of a larva. In bryozoans, the larva undergoes irreversible metamorphosis resulting in the formation of the ancestrula (see Zimmer and Woollacott 1977), the founding member of the colony, which through repeated budding develops into a reproductively mature colony (Fig. 1.1). Bryozoan colonies can be composed of thousands of individuals, termed zooids. Zooid polymorphisms occur in all species of bryozoans (see Silén 1977), although the general structure of a zooid is relatively conserved. In general, zooids are composed of an outer non-cellular membrane, the cuticle, which bounds a cellular membrane and together make up the cystid (McKinney and Jackson 1989).

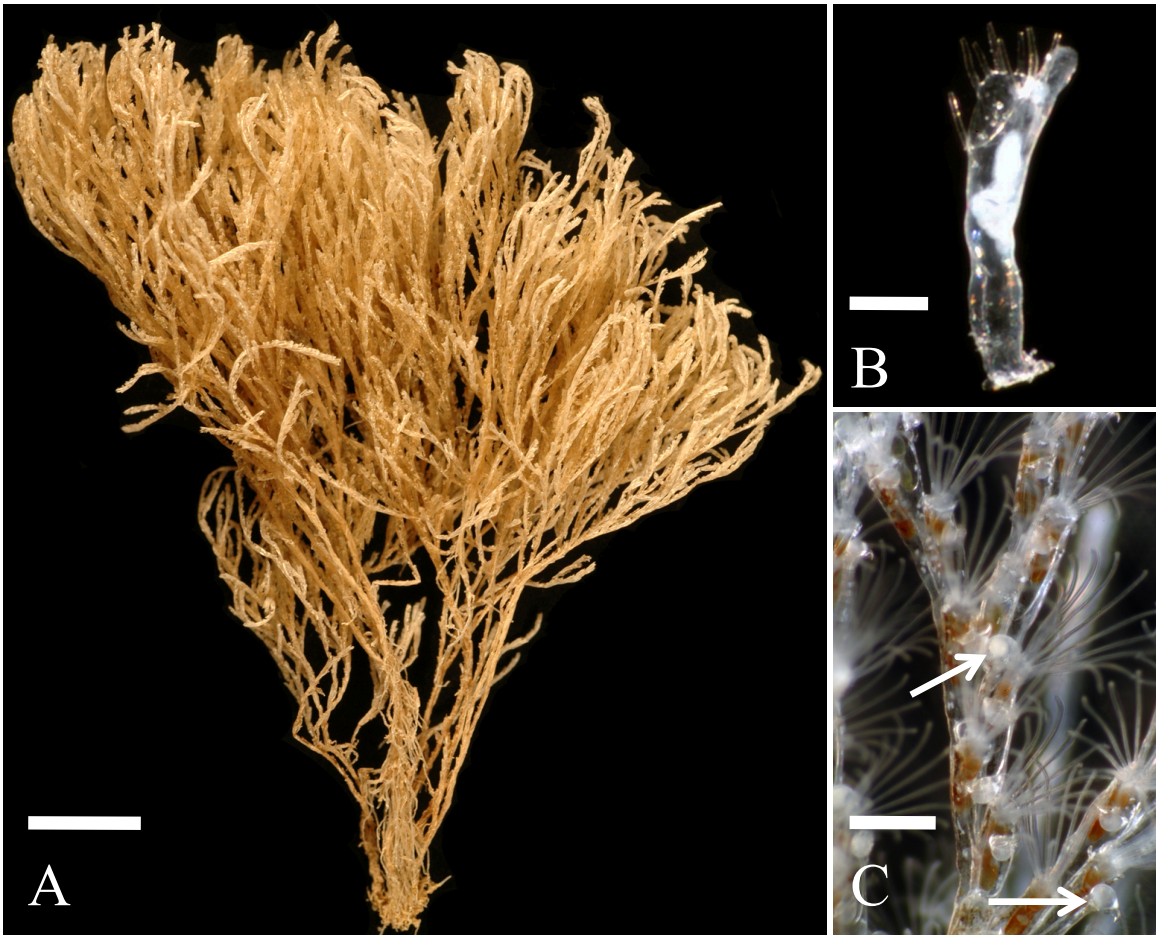

Figure 1.1. *Bugula stolonifera* Ryland, 1960. A) Erect, arborescent bryozoan that forms compact tufts, normally greyish-white to straw-colored. Scale bar = 1 cm. B) Founding member of the colony, termed the ancestrula. Note the characteristic spines centered around the frontal membrane. Scale bar = 200 μm. C) Branch of a reproductively mature colony showing biserially arranged zooids with lophophores extended. Also visible are avicularia (lower right) and a brooded embryo (center). Scale bar = 350 μm.

The cystid surrounds the coelomic cavity, which contains the various tissues specific to particular zooids. The types of zooids within the colony can be loosely divided into two broad categories: feeding and non-feeding (Silén 1977).

The fundamental zooid common to all species of bryozoans is the feeding zooid, termed the autozooid (Levinsen 1902), which consists of the cystid and a fully formed polypide. The cystid is made up of the extracellular outerwall of the zooid, an epidermal

layer, and a mesothelial layer, which may or may not be continuous. The polypide is made up of the lophophore, a hollow ring of tentacles centered on the mouth, and a U-shaped digestive tract. Polypides function primarily in feeding, but in many species are also responsible for the release of sperm to the water column (*e.g.*, Silén 1966; Bullivant 1967; Temkin 1994). Both the cystid and the lophophore contain coelomic compartments, with exchange of fluid occurring across an incomplete septum that separates the two compartments. It was originally thought that the cystid and the polypide were independent entities, such that the cystid was considered a "house", and the polypide simply resided in this "house" (see Ryland 1970). It is now understood that the cystid and the polypide are not only connected, but that for the current dominant group of bryozoans (the Gymnolaemata), the polypide buds from the cystid (*e.g.*, Faulkner 1933). Indeed, the formation and degeneration of multiple polypides throughout the life of a single cystid is common in many species (see Ryland 1976; Gordon 1977), and is evidenced by the formation of brown bodies within the body cavity (Fig. 1.2). Depending on the species, these brown bodies will either accumulate and remain within the cystid, or become incorporated within the gut of the newly developing polypide and ejected *via* defecation (Ryland 1976; Gordon 1977).

Non-feeding zooids are termed heterozooids and are characterized by lacking a fully functioning polypide (Silén 1977). While autozooids are common to all species of bryozoans, the possession of certain types of heterozooids varies depending on the species. Heterozooids perform various functions within the colony, including cleaning, reproduction, colony defense, and colony attachment (Silén 1977; McKinney and Jackson 1989). Particularly important for erect colonies, certain heterzooids can also function in

colony propagation. Specialized heterozooids termed kenozooids, which function in colony attachment, can bud autozooids (Fig. 1.2), thus allowing for the formation of genets and enabling a colony to spread to its surrounding area.

Figure 1.2. Cultured *Bugula stolonifera* colony. Visible in the proximal portion of the colony are regressed polypides, termed brown bodies (arrow). The root-like projection (stolon) emanating from the center of the colony is used primarily for attachment, but can bud zooids that can eventually develop into a distinct colony, genetically identical to the founding colony. Scale bar = 1.5 mm.

Classification

Bryozoans are divided into three classes: the Phylactolaemata, Stenolaemata, and Gymnolaemata. The inter-relationship of these three classes is uncertain, but recent phylogenetic analyses provide evidence that the phylactolaemates are the basal group, and the gymnolaemates and stenolaemates are sister groups (Waeschenbach et al. 2012).

The phylactolaemates are found exclusively in fresh water, and most species are characterized by possessing polypides with U-shaped lophophores. The zooids within a colony are monomorphic and share a continuous coelomic cavity (Hyman 1959). Phylactolaemates are also characterized by the production of statoblasts (see Mukai 1973), cell masses that can withstand environmental fluctuations and can serve as an overwintering body. When environmental conditions improve, the two valves of the statoblast separate resulting in the emergence of a polypide (Mukai et al. 1984). In contrast to the fresh water phylactolaemates, the stenolaemates are exclusively marine, and were the most abundant and diverse class of bryozoans through the Paleozoic and Mesozoic eras (Boardman and Cheetham 1987). Of the five orders of stenolaemates that dominated the paleontological record however, only the cyclostomates persisted beyond the Triassic (≈ 200 mya). One distinguishing characteristic found in this class is that stenolaemate polypides are enclosed by a membranous sac (McKinney and Jackson 1989). The lophophore is extended through contractions of muscles surrounding the sac. Additionally, with the exception of the Cinctiporidae (Boardman et al. 1992), cyclostomate bryozoans are thought to be polyembryonic (*e.g.* Harmer 1890, 1898; Robertson 1902; Borg 1926; Ryland 1970; Hughes et al. 2005). Polyembryony has not been documented to occur in phylactolaemate nor gymnolaemate bryozoans.

Whereas stenolaemate bryozoans dominated prior to the Triassic, by far the most abundant class of bryozoans presently are the gymnolaemates, a diverse group of animals with highly varied growth forms and reproductive strategies. Despite originating in the Upper Ordovician (≈ 450 mya), gymnolaemate bryozoans experienced low abundance until the occurrence of a massive species radiation in the Late Cretaceous (≈ 50 mya)

(Boardman and Cheetham 1987). This increased rate of speciation is thought to be due to the evolution of brooding and the origin of short lived, non-feeding larvae in this group, which could have minimized genetic exchange among populations (Taylor 1988). Most species in this class are marine, although some euryhaline and fresh water species are known to occur (*e.g.*, Potts 1884; Toriumi 1956, Menon and Nair 1972). One unifying character for this class is the presence of the funiculus, a tissue strand that is used for interzooidal communication and nutrient transfer among zooids (McKinney and Jackson 1989). Gymnolaemate bryozoans are divided into two orders: Ctenostomata and Cheilostomata. Ctenostomate bryozoans are uncalcified; the zooidal walls are membranous or gelatinous. Additionally, ctenostomates lack heterozooids except for kenozooids. In contrast, many species of cheilostomates possess high amounts of polymorphism within a colony (Silén 1977). The zooidal walls of cheilostomates are also calcified (McKinney and Jackson 1989). For many species in this order, the frontal membrane remains flexible, and this flexibility is utilized to extend the lophophore (Farre 1837). As muscles attached to the membrane contract, the membrane deforms increasing pressure in the coelom. This increase in hydrostatic pressure causes the lophophore to become rigid and extend. The lophophore is withdrawn by relaxing the membrane contraction muscles, thus decreasing the hydrostatic pressure in the coelomic cavity, and contracting the lophophore retractor muscle. For some species of cheilostomates, however, the frontal membrane is sufficiently clacified as to prevent the membrane from deforming. In these species, the lophophore is extended through the use of an ascus, an inflatable sac located in the coelomic cavity of the zooid. As the ascus is inflated with water, hydrostatic pressure of the coelom is increased, resulting in eversion of the

lophophore. Species possessing an ascus have been grouped as the Ascophora, although the ascus is now considered a polyphyletic character (*e.g.*, Dick et al. 2000; Gordon 2000; Tsyganov-Bodounov et al. 2009; Waeschenbach et al. 2012). Species lacking an ascus have until recently been grouped as anascans; a reclassification with new suborders within Cheilostomata has been proposed (*e.g.*, Tsyganov-Bodounov et al. 2009; Waeschenbach et al. 2012).

The placement of bryozoans in relation to other organisms in the tree of life has been controversial (see Hyman 1959; Ryland 1970). Initial confusion in categorizing this phylum may have stemmed from an inability to properly observe the individual constituents of a colony. Although a colony is macroscopic and can be easily viewed when attached to a substrate, individual zooids are microscopic. This lack of detailed observation led some early scientists to incorrectly identify these animals as plants. Indeed, the classification term bryozoan proposed by Ehrenberg (1831) is translated as "moss animal." Once correctly identified as animals, bryozoans were initially grouped with cnidarians, and then ascidians (see Hyman 1959 and references therein). It was not until Ehrenberg (1831) noted that bryozoan polyps had separate openings for the mouth and anus that bryozoans were thus distinguished.

The distinctness of the polypide structure in bryozoans was subsequently utilized to group these animals with phoronids and brachiopods. Phoronids, tube-dwelling vermiform animals, and brachiopods, shelled animals superficially resembling bivalve mollusks, both have body plans whereby individuals possess a U-shaped gut that terminates outside the lophophore (Hyman 1959). Hatschek (1888) was the first to propose this relationship and grouped these three phyla as Tentaculata. Hyman (1959)

proposed that this group be renamed Lophophorata, which has become the common vernacular when describing this group of animals.

The anatomical and embryological similarities shared among the lophophorates resulted in a widespread acceptance that these animals were closely related, although the placement of this group relative to other metazoans remained speculative. Each of the lophophorates exhibits both deuterstome and protostome characteristics during development (Hyman 1959), and these animals were thus considered separate from either lineage (see Field et al. 1988). The advent of molecular techniques, however, allowed for an exhaustive investigation of the relationship of the lophophorates relative to other metazoans, irrespective of confounding developmental and anatomical characteristics. An early molecular phylogenetic study analyzed partial 18S ribosomal RNA sequences and provided evidence that lophophorates allied with protostomes (Field et al. 1988). Halanych et al. (1995) analyzed complete 18S RNA sequences and found similar results, documenting that the lophophorates clustered within the protostomes, most closely related to the annelids and mollusks. This new clade was designated as Lophotrochozoa, as most animals within the clade possessed either a lophophore or a trochophore larva. Subsequent studies have supported the lophotrochozoan clade, also termed Spiralia (Giribet 2002), within the protostomes (*e.g.*, Passamaneck and Halanych 2004; Dunn et al. 2008; Helmkampf et al. 2008), providing evidence that bryozoans are protostome animals. The position of bryozoans within the lophotrochozoans, however, remains unclear (*e.g.*, Dunn et al. 2008). Interestingly, the relationship of bryozoans to phoronids and brachiopods has also become uncertain (*e.g.*, Cohen 2000; Passamaneck and Halanych 2006; Helmkampf et al. 2008). Recent evidence has suggested that bryozoans

align more closely with entoprocts and cycliophorans, comprising a clade termed Polyzoa (Hejnol et al. 2009), although this relationship is uncertain as well (*e.g.*, Jang and Hwang 2009; Paps et al. 2009). Hence, more work is required to elucidate the phylogenetic position of this enigmatic group of animals.

Reproduction

Not only has confusion surrounded the classification of bryozoans with respect to other metazoans, historically researchers have been confounded by the reproductive modes of these animals until only recently (see Silén 1966). At the level of the colony, all bryozoans are simultaneous hermaphrodites. As discussed by Hughes et al. (2009), no other Phylum of colonial organisms possesses such universal hermaphroditism. While the various environmental conditions that might initially select for hermaphroditism are well understood (*e.g.*, Ghiselin 1969), it remains unclear why no bryozoan species possesses exclusively gonochoristic colonies (see Hughes et al. 2009 and references therein).

Although bryozoan colonies are hermaphroditic, individual autozooids generally express gender sequentially, first maturing male, then female, gonads. Alternatively, gonochoristic zooids are known to occur in certain species (*e.g.,* Nielsen 1981; Dyrynda and Ryland 1982; Hughes and Hughes 1986). Because male and female reproductive structures occur within the same zooid in most species, it was once thought that bryozoans were exclusive self-fertilizers. For instance, Huxley (1856) reported observing a mature testis and ovum in zooids from several species of the genus *Bugula* and from *Scrupocellaria scruposa* (Linnaeus, 1758). Additionally, he documented that fertilization

occurs in the coelomic cavity, thus implying that the resulting embryo was a product of self-fertilization. Hyman (1959) noted that for many authors, self-fertilization was the rule for bryozoans, although for species with gonochoric zooids, cross-fertilization must occur. She also states that it was clear that fertilization occurred in the coelomic cavity of female zooids, but how sperm entered the maternal coelom was unknown. Although sperm entry had never been observed, she speculated that sperm could enter *via* an opening at the base of the lophophore, termed the supraneral pore or intertentacular organ (ITO) depending on the species.

Silén (1966) posited that cross-fertilization should occur in bryozoans, due to the potential deleterious effects associated with exclusive self-fertilization. For instance, selfing could result in increased homozygosity in individuals, leading to the retention of recessive deleterious alleles (Shields, 1982; Charlesworth and Charlesworth, 1987). The expression of these alleles could lead to significantly decreased survival, growth rate, and fecundity, culminating in substantially decreased fitness (Charlesworth and Charlesworth, 1987). Further, extensive self-fertilization could result in the complete loss of genes within populations, rendering these populations as a whole incapable of adapting to changes in environmental conditions (Maynard Smith, 1978). As a potential means of avoiding self-fertilization, Silén (1966) showed that for two species of the genus *Electra,* sperm were released through the tips of certain tentacles of the lophophore. Presumably, by releasing sperm to the water column, outcrossing would occur when adjacent colonies used this sperm for fertilization. This manner of sperm release was later demonstrated in a diverse array of ctenostomes and cheilostomes (Bullivant 1967; Strom 1969; Silén 1972), suggesting that spermcasting (Bishop and Pemberton 2006) was a reproductive

mode common to all bryozoans. Interestingly, the manner of sperm uptake by female zooids remained unclear, as did the potential for self-fertilization in these animals.

Elegant work conducted by Temkin (1994, 1996) examined fertilization in bryozoans, which led to a better understanding of the fertilization process in these animals. Working with *Membranipora membranacea*, an encrusting bryozoan that releases fertilized eggs to the water column for planktonic development, Temkin (1994) showed that fertilization occurred in the coelomic cavity of female zooids, although egg activation did not occur immediately following sperm-egg fusion. In this process, spermatozeugmata, packets of 32 or 64 sperm cells, released from the tips of certain tentacles of male zooids became entrained in the lophophore of a female zooid and entered the coelomic cavity through the ITO. After sperm-egg fusion, the fertilized egg was spawned to the water column through the ITO. Shortly after release, the fertilized egg was activated, resulting in the elevation of the fertilization envelope. The process of egg release induced significant deformation in the fertilized egg, a phenomenon that has been observed in other species as well (Silén 1945, Reed 1991). Delaying activation until after release ensures that the egg retains enough flexibility to survive the transfer process, although it remains unclear how polyspermy is prevented in these animals. Temkin (1996) extended his observations to nine species of ctenostome and cheilostome bryozoans with varying reproductive modes. Results from these observations documented that for all species, fertilization occurred in the coelomic cavity of maternal zooids and activation was delayed until eggs were released.

The aforementioned studies provide clear evidence of the fertilization process in bryozoans, documenting the uptake of sperm from the water column and internal

fertilization. Although this process appears to promote outcrossing, studies have shown that the entry point for sperm, the supraneural pore or ITO, does not appear to regulate sperm entry (*e.g.,* Dyrynda and King 1982; Temkin 1994). Rather, any conspecific sperm contacting a lophophore is transferred to the maternal coelom; hence the potential for self-fertilization persists. As previously discussed, only a few studies have directly examined selfing in bryozoans (*e.g.*, Maturo 1991; Temkin 1991; Hunter and Hughes 1993; Hughes et al. 2009), and the effects on offspring survival and reproductive fitness remain unclear.

Dispersal potential

As previously mentioned, Taylor (1988) asserted that the evolution of short-lived larvae in cheilostome bryozoans resulted in the massive species radiation observed in the fossil record, primarily by minimizing genetic exchange among populations. Studies examining larval physiology in brooding bryozoans support this assertion. For instance, Grave (1930) documented that the swimming period for *Bugula flabellata*[2] (Thompson in Gray, 1848) larvae was only approximately 4-6 hours. Short larval swimming durations have also been found in other brooding species (e.g., Brancato and Woollacott 1982; Woollacott et al. 1989; Orellana et al. 1996; Marshall and Keough 2003), suggesting restricted dispersal potential is common throughout this group. Further, although numerous physical and chemical factors have been shown to lengthen the larval phase of these short-lived anenteric larvae (see Lynch 1961), studies have documented significant deleterious effects associated with a protracted larval stage. Woollacott et al. (1989)

[2] The species used by Grave is believed to have been incorrectly identified and should be documented as *Bugula simplex* Hincks, 1886 (Ryland et al. 2011)

documented that prolonging the larval period beyond 6 hours for *B. stolonifera* larvae resulted in decreased juvenile survival and growth. Likewise, Wendt (1996, 1998) documented that an extended larval swimming period for *B. neritina* larvae causes a significant reduction in initiation and completion of metamorphosis, significantly reduced size of post-metamorphic individuals, and significantly decreased post-metamorphic growth and reproduction. For non-feeding larvae, prolonging the larval duration results in increased consumption of endogenous reserves, resulting in either decreased survival or significantly reduced fitness for post-metamorphic individuals. It has been suggested, however, that the uptake and utilization of dissolved organic matter (DOM) could provide non-feeding larvae with a nutrient source (see Jørgensen 1976), thus allowing for a protracted larval phase and increased dispersal. Although utilization of DOM has been shown to offset some of the costs associated with an extended larval swimming duration in *B. neritina* (Johnson and Wendt 2007), prolonged larval swimming in individuals with access to an abundant source of DOM still resulted in either decreased survival or significantly reduced fitness. Hence, the low dispersal potential for non-feeding larvae resulting from the short larval duration and the inability to cope with a protracted swimming period suggest that minimum genetic exchange should exist among populations of brooding bryozoans. On the other hand, recent evidence suggests that dispersal potential might be a poor indicator of actual distance achieved (see Weersing and Toonen 2009), and only a few studies have examined population connectivity in gymnolaemate bryozoans.

Large-scale studies comparing population connectivity in species with long-lived cyphonautes larvae versus species with short-lived coronate larvae have found that larval

period correlates strongly with population differentiation (*e.g.*, Watts and Thorpe 2006). For instance, Porter et al. (2002) sampled bryozoans from sites separated by 200-300 km and found greater differentiation in the brooding ctenostome *Alcyonidium gelatinosum* (Linneaus, 1761) compared to *A. mytili* Dalyell, 1848, a species with planktonic larval development. Fine-scale studies examining differentiation among populations are less conclusive. Hoare and co-workers (1999) used four polymorphic microsatellites to examine genotypic differentiation in several populations of *C. hyalina* and found no significant differences in populations separated by up to 8 km, despite this species possessing short-lived larvae (see Orellana et al. 1996). Conversely, Goldson et al. (2001) used Random Amplification of Polymorphic DNA to show genetic differentiation in samples of *C. hyalina* separated by only 10 m. Although rafting or anthropogenic dispersal cannot be discounted in contributing to the low differentiation among sites reported by Hoare et al. (1999), recent studies have documented greater than expected dispersal in other species with short-lived larvae (*e.g.*, Miller and Ayre 2008; Maier et al. 2009). For brooding bryozoans, the extent of larval dispersal and the potential for population connectivity remains unclear. Studies examining population genetics, on the scale of meters to hundreds of meters, are needed to determine if dispersal potential equates to realized larval dispersal in these species.

Thesis summary

In Chapter 2, I investigated the consequences of self-fertilization on offspring survival and reproductive fitness in *B. stolonifera*. Larvae from field-collected colonies (Eel Pond, Woods Hole, MA) were cultured through metamorphosis to reproductively

mature colonies either in the presence of one other colony or alone. Results documented that although selfing in this species is possible, colonies cultured on their own released significantly fewer larvae with significantly reduced rates of metamorphic initiation and completion, as compared to the control treatment. By transplanting offspring to the field for growth to reproductive maturity, additional deleterious effects from selfing were documented; no viable larvae were recovered from colonies deriving from the solitary treatment. In contrast, 1030 larvae were collected from the control treatment, and these individuals were found to have extremely high rates of metamorphic initiation (99%) and completion (97%). Overall, selfed larvae not only had significantly decreased chances of survival, but those that did survive did not successfully reproduce.

In Chapter 3, I developed and utilized a microsatellite library to examine the genetic make-up of conspecific aggregations in *B. stolonifera*. Additionally, as conspecific larvae routinely metamorphose on adult colonies, I investigated the possibility that larvae might select or avoid their maternal colony. Adult colonies and their attached individuals were genotyped and compared to assess genetic relatedness within and among these groups relative to the overall genetic variability of the sampling site. No significant genetic differentiation was found between any groups, documenting that a group containing an adult colony and its attached individuals had as much genetic variability as was found for the entire sampling site. Parentage-exclusion analyses showed that the vast majority of attached individuals (>93%) could not have derived from the colony on which they were attached. Kinship analyses showed that approximately 63% of attached individuals shared less than a half-sibling relationship.

These results suggest that a colony's nearest neighbors are not composed of siblings, and thus, larval settlement preference can minimize selfing in this species.

In Chapter 4, I investigated whether the high larval mixing within an aggregation of *B. stolonifera* documented in Chapter 3 also results in high mixing among aggregations. Significant genotypic differentiation was found among most sites, suggesting limited genetic connectivity among aggregations, even those separated by only 100 m. This study was extended to determine if low levels of genetic mixing among sites throughout the reproductive season could result in increased homogeneity among aggregations. Results from these latter analyses documented that genetic mixing could result in increased homogeneity between some aggregations. Results from inter-annual comparisons within sites, however, suggest that any potential homogeneity achieved throughout the reproductive season will likely be lost by the beginning of the next reproductive season due to the annual cycle of colony die-off and re-growth experienced by *B. stolonifera* colonies.

In Chapter 5, I documented the first occurrence from the western Atlantic Ocean of the widely distributed arborescent bryozoan *Tricellaria inopinata* d'Hondt and Occhipinti-Ambrogi, 1985. At the time of its first observance in Eel Pond in September 2010, *T. inopinata* colonies had already formed dense conspecific aggregations at some collection sites, despite the presence of several other arborescent bryozoans. Sites were monitored throughout 2011 to track the success of this introduction, and to assess the reproductive timing of *T. inopinata* in Eel Pond. *Tricellaria inopinata* colonies were found to be reproductive from early June through December 2011, a duration that exceeded other arborescent Eel Pond bryozoans. Timing and overall rates of

metamorphic initiation and completion by offspring of *T. inopinata* were comparable to those of another dominant arborescent bryozoan, *B. stolonifera*, suggesting that *T. inopinata* will likely persist in Eel Pond following this introduction. Finally, I provided taxonomic details to aid in identifying these animals, considered the potential mode of transport, and discussed the potential ecological implications resulting from this introduction.

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Chapter 2

Effects of selfing on offspring survival and reproduction in a colonial simultaneous hermaphrodite (*Bugula stolonifera*, Bryozoa)

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Effects of Selfing on Offspring Survival and Reproduction in a Colonial Simultaneous Hermaphrodite (*Bugula stolonifera*, Bryozoa)

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Abstract. Understanding the consequences of selfing in simultaneous hermaphrodites requires investigating potential deleterious effects on fitness at all stages of life. In this study, I examined the effects of selfing throughout the life cycle of the marine bryozoan *Bugula stolonifera*, a colonial simultaneous hermaphrodite. In 2008, larvae from field-collected colonies were cultured through metamorphosis to reproductively mature colonies either in the presence of one other colony, the paired treatment, or alone, the solitary treatment. Results demonstrated that selfing in this species is possible, in that colonies in the solitary treatment produced viable larvae that successfully completed metamorphosis. On average, however, these colonies released significantly fewer larvae, which experienced reduced rates of metamorphic initiation and completion compared to the paired treatment. These experiments were extended in 2009, when metamorphs from colonies reared in the solitary ($n = 58$) and paired ($n = 61$) treatments were transferred to the field for growth to reproductive maturity and then brought back to the laboratory for larval collection. Results revealed additional deleterious effects associated with selfing, as no viable larvae were recovered from colonies deriving from the solitary treatment. In contrast, offspring from the paired treatment released 1030 larvae and 99% initiated metamorphosis, 97% of which completed metamorphosis. Overall, selfed larvae not only had significantly decreased chances of survival, but those that did survive did not successfully reproduce.

Introduction

For over a century, researchers have been interested in the potential for self-fertilization in simultaneous hermaphrodites (see reviews by Jain, 1976; Jarne and Charlesworth, 1993; Jarne and Auld, 2006). Specifically, studies have centered on the distinct benefits selfing can confer as compared to exclusive out-crossing (Maynard Smith, 1971; Charlesworth, 1980; Lively and Lloyd, 1990). Sexual reproduction requires the fusion of gametes, with each gamete containing 50% of its parent's genome. This reduction in parental legacy has been termed the cost of meiosis (Williams, 1975) and can be avoided by selfing *via* self-fertilization, the fusion of gametes from the same individual; by automixis, the activation of a meiotically divided cell (see Mogie, 1986); or by apomixis, a process particular to angiosperms and gymnosperms where seeds form without the need of meiosis and fertilization (see Bicknell and Koltunow, 2004). These processes can also alleviate some of the risks associated with sex, including finding an appropriate mate in copulating species and dilution of gametes in spawning species. Alternatively, it is thought that selfing can have deleterious effects such as decreased survival, growth rate, and fecundity, culminating in substantially decreased fitness or inbreeding depression (Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987). This decrease in fitness could potentially stem from increased homozygosity, leading to retention of recessive deleterious alleles (Shields, 1982; Charlesworth and Charlesworth, 1987), or from the loss of genes in selfing populations, rendering the population as a whole incapable of adapting to changes in environmental conditions (Maynard Smith, 1978). These deleterious consequences associated with selfing were recently demonstrated empirically for the highly selfing nematode *Caenorhabditis elegans* (Morran *et al.,* 2009). The authors documented that after 50 generations, increased mutation rate coupled with a change in environmental condition led to significantly reduced fitness

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in an obligate selfing strain of *C. elegans*, as compared to outcrossed controls. Other studies have shown that inbreeding depression can occur as rapidly as within the next generation (*e.g.*, Charlesworth *et al.*, 1994; Cohen, 1996; Escobar *et al.*, 2007). Thus the evolution and maintenance of selfing is thought to depend on the trade-offs of various costs and benefits (Lande and Schemske, 1985).

Although selfing has historically been more heavily investigated in plants (Jain, 1976), and in particular in angiosperms (*e.g.*, Darwin, 1876; Bicknell and Koltunow, 2004; Barringer, 2007), these processes in metazoans have recently received increased attention (Jarne and Auld, 2006). For dioecious organisms, automixis has been documented in some animals, including rotifers (Stelzer, 2008), bivalves (Fóighil and Thiriot-Quiévreux, 1991), beetles (Moore *et al*., 1956), grasshoppers (Atchley, 1978), wasps (Beukeboom and Pijnacker, 2000), and vertebrates (Adams *et al.,* 2003; Watts *et al*., 2006). Selfing is widely reported in simultaneous hermaphrodites, including cnidarians (Bucklin *et al*., 1984; Bassim *et al*., 2002; Sherman, 2008), molluscs (Meunier *et al*., 2004; Escobar *et al*, 2007; Smolensky *et al*., 2009), annelids (Finley *et al*., 2001; Méndez, 2006), flatworms (Christen and Milinski, 2003; Lagrue and Poulin, 2009), and ascidians (Ryland and Bishop, 1993; Cohen, 1996; Jiang and Smith, 2005; Manríquez and Castilla, 2005).

Conclusive evidence demonstrating the ability to self in simultaneous hermaphrodites comes from laboratory-based studies, wherein individuals are either maintained in isolation (*e.g.*, Sabbadin, 1971) or gametes from a simultaneous hermaphrodite are procured (Bassim *et al*., 2002), and the effects of selfing examined. Alternatively, researchers have utilized genetic techniques to examine selfing in the field. Bucklin *et al.* (1984) collected 25 individuals of the brooding sea anemone *Epiactis prolifera* and, using allozyme electrophoresis, found that genotype frequencies of the brooded offspring were consistent with selfing. Dupont *et al.* (2007) utilized microsatellite markers to investigate selfing in two populations of the brooding ascidian *Corella eumyota* and found significant rates of selfing in both populations. It is thought that for species with limited dispersal potential, including those that brood embryos, inbreeding might be more prominent (Knowlton and Jackson, 1993). For these species, this could eventually lead to increased tolerance of inbreeding, culminating in populations that are capable of selfing with little to no inbreeding depression (Lande and Schemske, 1985; Uyenoyama, 1986).

The phylum Bryozoa is dominated by species that brood embryos and release short-lived larvae that have low potential for dispersal (*e.g.*, Ström, 1977; Zimmer and Woollacott, 1977). For example, larvae of *Bugula* spp. will usually initiate metamorphosis within 1–4 h after release (*e.g.,* Woollacott *et al*., 1989; Wendt and Woollacott, 1999). At the level of the colony, bryozoans are simultaneous hermaphrodites; individual autozooids, however, generally express gender sequentially, first maturing male, then female, gonads. Alternatively, gonochoric zooids are known to occur in certain species (*e.g.,* Dyrynda and Ryland, 1982; Hughes and Hughes, 1986). Because male and female reproductive structures occur within the same zooid, it was once thought that bryozoans were exclusive self-fertilizers (see Silén, 1966). Elegant work detailing bryozoan fertilization led researchers to conclude that these animals were, at least, capable of out-crossing. For instance, Silén (1966) showed that for two species of the genus *Electra,* sperm were released through the tips of certain tentacles of the lophophore. By "spermcasting" (Bishop and Pemberton, 2006), sperm can presumably be transferred to a different colony for fertilization, although the uptake of this sperm by another zooid of the same colony may also be possible. This manner of sperm release was later demonstrated in such a diverse array of other ctenostome and cheilostome bryozoans (Bullivant, 1967; Ström, 1969; Silén, 1972) that Silén (1972) concluded it was likely that cross-fertilization occurred in all groups of bryozoans.

Only a few studies have investigated selfing in bryozoans (see review by Ostrovsky, 2008) despite the more than 5500 extant species. For instance, Maturo (1991) found that colonies from six brooding species reared in isolation were able to release larvae, and Temkin (1991) found that isolated colonies of *Membranipora membranacea* were able to release embryos that developed into larvae. Working in natural populations, Yund and McCartney (1994) and McCartney (1997) used allozyme electrophoresis to provide evidence of selfing in populations of *Celleporella hyalina* from Walpole, Maine. These studies suggest that the ability to self is widespread in the gymnolaemate bryozoans, but the consequences of selfing to offspring fitness remain unclear. Interestingly, a series of studies examining reproduction in *C. hyalina* found that colonies from certain populations cultured in isolation released larvae that were unable to initiate metamorphosis (Hunter and Hughes, 1993a; Hoare and Hughes, 2001), whereas isolated colonies from other populations were able to self with no measured decrease in offspring survival (Hughes *et al*., 2002b, 2009). It is unknown from these latter studies if selfed progeny were able to reach reproductive maturity.

In this study, I report the results from experiments investigating the effects of selfing in the cheilostome *Bugula stolonifera* Ryland 1960. This species is cosmopolitan in temperate and tropical waters (Rodgers and Woollacott, 2006) and is suitable for this study for several reasons: (1) I am able to culture *B. stolonifera* from larvae through reproductively mature colonies in the laboratory, (2) brood chambers are distinct in this species, making it possible to determine onset of reproduction in cultured colonies, and (3) brood chambers are transparent, which allows for accurate counts of brooded embryos in gravid colonies. By

culturing both solitary colonies and colonies growing adjacent to a conspecific (paired treatment), I examine the possibility of selfing and the subsequent effects on offspring survival and fecundity.

Materials and Methods

Experimental procedures

Sexually mature colonies of *Bugula stolonifera* were collected from the sides of floating docks in Eel Pond, Woods Hole, Massachusetts, in September 2008 and July 2009. Colonies were maintained overnight in the laboratory in complete darkness in a 38-l glass aquarium equipped with a power filter providing water flow and aeration. To induce larval release, colonies were removed from the aquarium, placed in 1.5-l glass bowls containing unfiltered Eel Pond seawater (UFSW), and exposed to fluorescent light. Larvae appeared about 15 min after exposure to light, and within an hour hundreds of larvae had aggregated on the illuminated side of the dish. Groups of 8 to 75 larvae were pipetted into 35-mm polystyrene dishes (BD Falcon #353001), which were then covered and transferred to the dark to facilitate larval attachment. No attempt was made to exclude closely related larvae from these experiments. To estimate the overall health of the collected colonies, the percentage of larvae initiating and completing metamorphosis was assessed. Percent metamorphic initiation was assessed after 4 h, and larvae not initiating metamorphosis were removed. Polystyrene dishes with attached metamorphs were then transferred to covered, 150-ml beakers containing 125 ml of UFSW and placed on a continuously oscillating orbital shaker to keep the water within the beaker mixed. The alga *Rhodomonas* sp. (Bigelow Laboratory CCMP757) was added the following morning, ensuring that ancestrulae could commence feeding immediately at completion of metamorphosis. Polystyrene dishes were maintained in a vertical position within the beaker to prevent the build-up of settled food and waste products adjacent to the growing individuals. Percent metamorphic completion was assessed about 72 h after metamorphic initiation, and individuals were then selected for the selfing experiment.

To examine the effects of selfing on offspring survival, colonies were cultured either in isolation, the solitary treatment, or in the presence of one other conspecific, the paired treatment. A total of 58 colonies were cultured in 2008 (solitary $n = 16$, paired $n = 10$) and 2009 (solitary $n = 22$, paired $n = 10$). Cultured colonies were maintained at 24 °C in a constant temperature room with a 16 h:8 h light/dark cycle, and fed *Rhodomonas* sp. twice daily at a final concentration of at least 10,000 cells ml^{-1} (Winston, 1976; Hunter and Hughes, 1993b). Colonies were maintained in UFSW on the orbital shakers and were cleaned and inspected daily. To prevent contamination from sperm contained within the field-collected water, water from the Eel Pond was aged for at least one week prior to use. Colonies were cleaned with a soft artist's brush daily to remove attached algal cells, and care was taken to prevent cross-contamination among beakers. Only one polystyrene dish was removed at a time. Dishes were removed with forceps when placed under the dissecting scope, and then transferred immediately after inspection and cleaning to a new beaker. Forceps and all surfaces were sterilized with 95% ETOH prior to examination of the next individual. Beakers remained covered the entire time to prevent contamination *via* splashing, and each beaker had a designated artist's brush for cleaning. Growth, as the number of bifurcations per colony, and onset of reproduction, as the presence of brood chambers, were assessed daily.

About one week after a filled brood chamber was observed, the total number of chambers and the number of brooded embryos per colony were recorded. To minimize larval release, reproductively mature colonies were moved to a dark constant temperature room also held at 24 °C. The feeding regime remained the same for these colonies. Colonies that had not reached reproductive maturity or had not brooded embryos remained on the original light/dark cycle until they became gravid. Larval releases were conducted every morning for 1 week as previously described, except that polystyrene dishes with attached colonies were placed in glass Stender dishes containing 20 ml UFSW. The smaller glass dish and volume of water facilitated larval collection. Larval release was allowed to continue for 2 h, after which colonies were cleaned, placed in new UFSW, and returned to the dark room. Collected larvae were transferred to clean polystyrene dishes and placed in the dark. Percent metamorphic initiation was determined after 4 h. The dishes were then flooded, ensuring that all settled metamorphs were submerged, and percent metamorphic completion was determined after 72 h.

Culturing experiments conducted in 2008 demonstrated that *B. stolonifera* colonies reared in isolation produced viable larvae that successfully completed metamorphosis. In summer 2009 these experiments were extended by transferring metamorphs from colonies reared in the solitary and paired treatments back to the field to examine the effects of selfing on offspring survival and reproductive fitness. Larvae from cultured individuals were allowed to settle on polystyrene weighing dishes (VWR #12577-005). After 4 h, percent metamorphic initiation was assessed, and unattached larvae were removed from the weighing dish. Metamorphs were marked by circling their position on the weigh boat to aid in identifying these individuals, thus preventing confusion with newly settled ancestrulae that might attach after dishes were transferred to the field. Weighing dishes were clipped into plastic binders, which were then affixed within a rectangular acrylic plastic chamber. The chambers (15.3 cm (H) × 7.6 cm (W) × 5.7 cm (D)) contained grooved sides, allowing the binders to slide into the

grooves. A total of 5 weighing dishes were placed in each chamber, with 2.5 cm (vertical distance) between dishes. Previous tests showed that predation on newly settled individuals within these chambers was not prominent in Eel Pond, so protective screening was not used and the dishes were held in place simply by using several large rubber bands. Generally, *B. stolonifera* larvae will complete metamorphosis 24–48 h after initiation. Therefore, metamorphs (paired: $n = 61$; solitary: $n = 59$) were transferred to Eel Pond, Woods Hole, Massachusetts, within 24 h of settlement, enabling ancestrulae to feed immediately upon completion of metamorphosis. Chambers were suspended from the WHOI-MBL pier in Eel Pond at a site adjacent to numerous established *B. stolonifera* colonies. The chambers were weighted at the bottom to maintain vertical orientation within the water column and were suspended about 1 m below the surface. Within the chamber, colonies were oriented downward to prevent the build-up of sediment on the weigh boat. Colonies were inspected twice weekly for survival and onset of reproduction, and alien juveniles were removed from the weighing dish. About 2 weeks after the appearance of the first filled brood chamber, colonies were returned to the laboratory for larval collection. Larval release was conducted as previously described, except that weigh boats with attached colonies were placed in 250-ml bowls containing UFSW prior to exposure to light. The total number of larvae released and the total number of these individuals that initiated and completed metamorphosis were used to determine reproductive fitness.

Statistics

The effect of treatment (solitary or paired) on the ability of cultured colonies to reach reproductive maturity was investigated using Fisher's Exact Test. Significant differences in all other measured variables between the experimental treatments within each year were investigated using a one-way ANOVA with the statistical package Minitab ver. 15. The measured variables were as follows: time to reach reproductive maturity, colony size at reproductive maturity, total number of brood chambers per colony, percentage of filled brood chambers per colony, number of larvae released per treatment, number of larvae initiating metamorphosis per treatment, and number of individuals completing metamorphosis per treatment. Because there were two individuals in each beaker in the paired treatment and one individual in each beaker in the solitary treatment, the number of larvae released and the number of individuals initiating and completing metamorphosis from the paired treatments were halved prior to analyses to correct for this discrepancy. Prior to conducting the ANOVA, all data sets were initially examined for normality and equal distributions. Data sets failing these tests were transformed to meet these assumptions using either the squared, square root, or 4^{th} root transformation, depending on the data set. The time to reach reproductive maturity from 2009 continued to fail these tests, so the untransformed data were subjected to the nonparametric Kruskal-Wallis analysis, with condition as the factor. These data are presented as medians; all other data are presented as untransformed means $\pm$ 1 standard error.

Results

Fitness of field-collected parental colonies

In 2008, 375 larvae were sampled from field-collected colonies. Of these, 342 (91.2%) initiated metamorphosis, and 330 (96.5%) of those completed metamorphosis. In 2009, 950 larvae were collected, 884 (93.1%) initiated metamorphosis, and 871 (98.5%) of those completed metamorphosis. Due to the high rates of metamorphic initiation and completion, parental colonies were considered healthy, and individuals successfully completing metamorphosis were selected for the selfing experiments.

Colony growth in culture

Bugula stolonifera colonies were amenable to culturing, as there was no mortality in either year. For both years, growth rates varied between the treatments such that, on average, solitary colonies were significantly larger at reproductive maturity than colonies cultured in pairs (Fig. 1; Table 1). Although solitary colonies were significantly larger at the time of assessment, there were no significant differences in time to reach reproduction between the conditions in either year (Fig. 1; Table 1).

Effect of selfing on brooding and larval fitness

In 2008, 12 of 16 solitary colonies and 10 of 10 colonies cultured in pairs reached reproductive maturity, demonstrated by the production of brood chambers ($P = 0.122$). On average, there was no significant difference between treatments in the total number of brood chambers developed per colony (Tables 1 and 2). In contrast, colonies cultured in pairs had a significantly higher percentage of filled brood chambers at the time of assessment (Tables 1 and 2), as well as a significantly higher total number of larvae released ($P = 0.030$) and total number of offspring initiating ($P = 0.016$) and completing ($P = 0.042$) metamorphosis compared to the solitary treatment (Fig. 2). Additionally, larvae released from solitary colonies experienced reduced rates of metamorphic initiation (33.1%) compared to larvae from the paired treatment (43.7%). Of those that initiated, offspring from solitary colonies also experienced reduced rates of metamorphic completion (81.1%) compared to the paired treatment (97.7%) (Fig. 2).

In 2009, 20 of 22 solitary and 10 of 10 paired colonies reached reproductive maturity ($P = 0.466$). Here, colonies cultured in the paired treatment on average had significantly

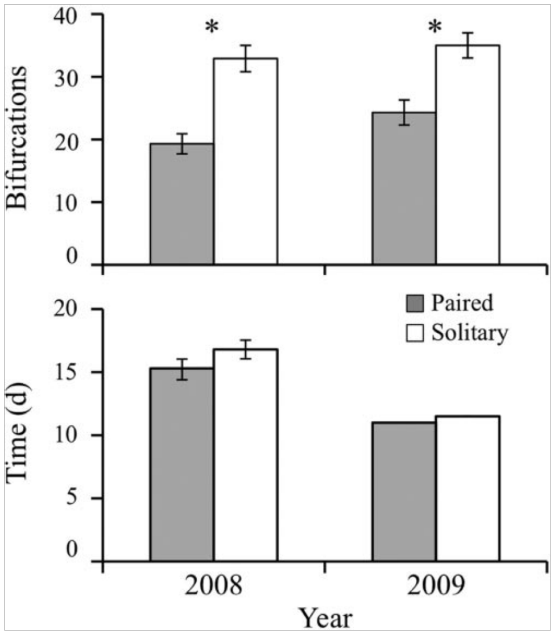

Figure 1. Average size at and time to reach reproductive maturity for colonies cultured in the paired and solitary treatments. Time to reach reproductive maturity data in 2009 analyzed by Kruskal-Wallis test (data presented as medians, error bars not calculated); all other data analyzed by one-way ANOVAs. Significant differences between conditions within each year indicated by an asterisk ($P \leq 0.002$). Bars = 1 S.E.

Table 2

Number of brood chambers and percentage of filled brood chambers per colony for each treatment

Year	Treatment	Brood chambers	*P* value	Filled brood chambers (%)	*P* value
2008	Paired	42.6 (±12.0)	0.587	72.2 (±3.0)	0.003
	Solitary	34.7 (±12.1)		38.4 (±6.4)	
2009	Paired	111.9 (±16.7)	0.001	78.3 (±3.3)	0.015
	Solitary	52.3 (± 8.6)		59.2 (±4.9)	

The total number of brood chambers and percentage of filled brood chambers were assessed for each colony about one week after observing the first brooded embryo. Values are means ± 1 S.E. Differences were analyzed with a one-way ANOVA.

more brood chambers per colony as well as a significantly higher percentage of filled brood chambers compared to solitary colonies (Tables 1 and 2). As in 2008, paired colonies also had a significantly higher number of larvae released ($P < 0.001$) and a higher number of individuals initiating ($P < 0.001$) and completing metamorphosis ($P < 0.001$) compared to colonies cultured in the solitary treatment (Fig. 2). Additionally, as in 2008, larvae released from solitary colonies in 2009 experienced reduced rates of metamorphic initiation (71.3%) and completion (65.2%) compared to those cultured in pairs (rate of initiation = 86.7%; rate of completion = 95.4%) (Fig. 2).

Table 1

Effect of culturing treatment on the time in days to reach reproduction, number of bifurcations at onset of reproduction, number of brood chambers developed per colony, and percentage of filled brood chambers per colony

Year	Measurement	Source	df	MS	F_{stat}	*P* value
2008	Time	Treatment	1	12.20	1.68	0.209
		Error	20	7.26		
	Bifurcations	Treatment	1	1037.2	23.99	<0.001
		Error	20	43.2		
	Total brood chambers	Treatment	1	3.01	0.30	0.587
		Error	20	9.88		
	Filled brood chambers	Treatment	1	0.6247	11.08	0.003
		Error	20	0.0564		
2009	Time	Treatment	1	–	–	0.358
	Bifurcations	Treatment	1	756.1	11.23	0.002
		Error	28	67.3		
	Total brood chambers	Treatment	1	23721	12.48	0.001
		Error	28	1901		
	Filled brood chambers	Treatment	1	0.3449	6.67	0.015
		Error	28	0.0517		

Time to reach reproduction in 2009 analyzed by the Kruskal-Wallis nonparametric test ($H = 0.84$; MS and F_{stat} not calculated); all others analyzed by a one-way ANOVA.

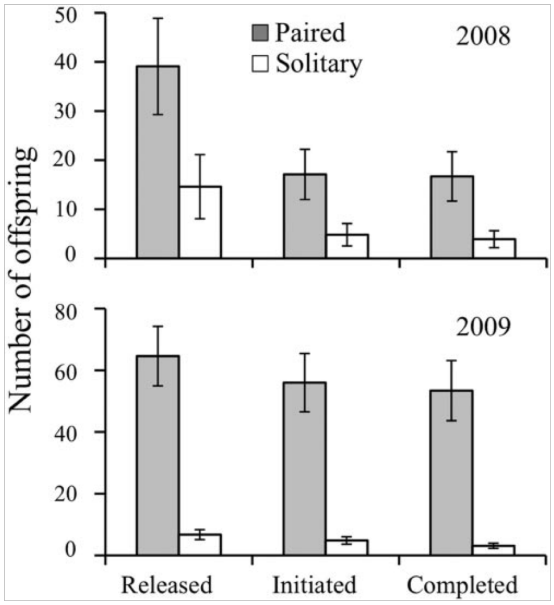

Figure 2. Average number of larvae released and average number of individuals initiating and completing metamorphosis per treatment. All paired data were halved to adjust for the presence of two individuals per treatment in this condition. Significant differences existed within each year between treatments for all measured variables (one-way ANOVA; $P < 0.05$). Bars = 1 S.E.

Effect of selfing on offspring reproductive fitness

A total of 61 metamorphs from the paired treatments were transferred to Eel Pond the morning after larval attachment, 59 (96.7%) of which were found to complete metamorphosis when examined 3 days later. As colonies from the paired treatments routinely released numerous larvae, only one release event was required to collect sufficient larvae (>50) for this experiment. The larvae from the five cultured pairs were allowed to settle on five polystyrene weighing dishes ($n = 4–17$ individuals per dish), and the dishes were affixed within one acrylic plastic chamber. These colonies were inspected twice weekly; they grew well and appeared healthy. Neither mortality nor predation was observed on any weighing dish while the colonies could be tracked individually (≈15 days post-settlement). About 12 days post-settlement the colonies on 4 of 5 dishes had grown to sufficient size to begin overgrowing each other, and by 15 days were so intertwined that distinguishing individual colonies became impossible. The remaining dish had only three individuals attached, and these could be followed directly. By day 12 post-settlement, individuals on all dishes had developed brood chambers, and by day 15 had commenced brooding embryos. Although the colonies could not be tracked individually, the dishes were monitored over the next 2 weeks as previously stated, and any newly settled ancestrulae were removed. Sixteen days after brooded embryos were observed, colonies were transferred back to the laboratory for larval release, which was conducted over the next 4 days. Colonies were found to release numerous larvae, with metamorphic initiation and completion rates approximating the rates found in field-collected colonies (Table 3). Colonies were then removed from the weighing dishes and assessed for survival and reproductive maturity. Of the 59 colonies, 56 (94.9%) were recovered, and all retained brooded embryos at the time of removal.

For the solitary treatments, 58 metamorphs were transferred to Eel Pond, 37 (63.8%) of which had completed metamorphosis when examined 3 days later. In contrast to colonies from the paired treatments, few larvae were collected from solitary colonies during a single release event. Therefore, four release events were required to collect approximately the number of larvae collected from the paired treatment colonies. The larvae from 14 different solitary colonies were settled on 19 weighing dishes ($n = 1–8$ individuals per dish), which were affixed in four acrylic plastic chambers. Due to the fewer number of offspring per dish as compared to the paired treatment, all individuals could be followed directly. Colonies were inspected twice weekly, and growth was found to vary greatly. By day 12 post-settlement, growth varied from ancestrulae with no buds to colonies approximating the size of those from the paired conditions. Additionally, by this time, 17 of the 37 individuals had been lost. Prior to their dislodgement from the weighing dish, all of these individuals were observed to be either simply ancestrulae with a regressed polypide or ancestrulae with but a few budded zooids. Of the 20 surviving colonies, 13 reached reproductive maturity. By day 23 post-settlement, all reproductively mature colonies brooded embryos. After an additional 14 days, these colo-

Table 3

Results of one-way ANOVA examining the effect of culturing treatment on total larvae released, total number of individuals initiating metamorphosis, and total number completing metamorphosis

Year	Measurement	Source	df	MS	F_{stat}	P value
2008	Released	Treatment	1	31.03	5.82	0.030
		Error	14	5.33		
	Initiated	Treatment	1	18.57	7.51	0.016
		Error	14	2.47		
	Completed	Treatment	1	10.18	5.27	0.042
		Error	11	1.93		
2009	Released	Treatment	1	6.479	49.98	<0.001
		Error	22	0.130		
	Initiated	Treatment	1	7.999	29.87	<0.001
		Error	22	0.268		
	Completed	Treatment	1	10.933	28.70	<0.001
		Error	22	0.381		

Table 4

Results from investigating survival and fecundity of offspring from colonies cultured in each treatment

Treatment	Transferred metamorphs	Recovered colonies	Reproductive colonies	Larvae released	Initiated metamorphosis	Completed metamorphosis
Paired	61	56	56	1030	1017 (98.7%)	986 (97.0%)
Solitary	58	20	13	3	0	–

Sexually mature colonies were collected from the field (Eel Pond, Woods Hole, MA) for larval release 14 d after brooded embryos were observed. Percent initiated are of total released; percent completed are of total initiated.

nies were transferred back to the laboratory for larval release, which was conducted over the next 3 days. These 13 colonies released a total of three larvae, none of which initiated metamorphosis (Table 4). In addition, during larval release, several aborted embryos were found at the base of the weighing dish, underneath the attached colonies. At the completion of the larval release period, only 2 of the 13 colonies still contained brooded embryos. For these colonies, 3 out of 75 and 2 out of 92 brood chambers were filled.

Discussion

The ability to self could convey distinct advantages compared to dependence on a mate for outcrossing (Maynard Smith, 1971; Charlesworth, 1980; Lively and Lloyd, 1990). Previous work directly examining selfing in bryozoans has shown that selfing is possible in several different species (Maturo, 1991; Temkin, 1991; Hughes *et al.*, 2009), but the consequences for survival and reproductive fitness have rarely been investigated. Here I show that selfing in *Bugula stolonifera* results in the release of viable offspring, but these individuals experience a significant reduction in fitness. As compared to outcrossed controls, this is expressed as significantly fewer larvae released, reduced rates of metamorphic initiation and completion, and decreased survival and fecundity. Indeed, no viable larvae were collected from reproductively mature selfed colonies.

Effect of a conspecific on reproduction

For *B. stolonifera* the presence of a conspecific did not induce an earlier onset of reproduction, nor did solitary colonies delay reproduction (Fig. 1; Table 1). These results demonstrate that this species is capable of reaching reproductive maturity without extrinsic cues. Previous studies examining the onset of reproduction in bryozoans have found that embryonic effects (genetic control or maternal contributions) and external cues can be responsible for reproductive timing, depending on the species. Working with the encrusting species *Membranipora membranacea*, Harvell and Grosberg (1988) found that conspecific crowding and colony damage resulted in an earlier onset of reproduction. Additionally, Harvell and Helling (1993) found that damage to one side of a colony of *M. membranacea* induced earlier onset of reproduction in adjacent zooids than in zooids on the undamaged side of the same colony, showing within-colony variation in reproductive timing. In contrast, Keough (1989a) collected offspring from *B. neritina* colonies growing in two areas, one with early reproductive onset and one with late, and found through a common garden growth experiment that reproductive patterns follow the patterns of the parental populations. That there was no significant difference in time to reach reproduction between treatments for my study supports Keough's findings, suggesting that for *Bugula* spp. the time to reach reproduction is determined prior to larval release. This pattern is not uniform within Bryozoa, however; onset of reproduction, as well as potential within-colony variation in reproductive timing, needs further investigation for other genera.

Although the presence of a conspecific did not significantly affect reproductive timing in *B. stolonifera*, it does appear to have had an effect on energy directed toward reproduction. In 2009, paired colonies were significantly smaller than solitary colonies, yet they had significantly more brood chambers (Tables 1 and 2). Although not significant, the data from 2008 trended similarly. This increase in brood chamber development in paired colonies suggests increased resource allocation toward the production of female zooids. Hence, the potential for outcrossing could lead to greater female investment. Sex allocation theory for simultaneous hermaphrodites predicts that selfing and mating groups of two should result in decreased production of males and increased production of females (Charnov, 1982; Fischer, 1984). As the number of individuals in the mating group increases, male allocation should increase due to sperm competition. That the solitary colonies produced fewer brood chambers suggests that the paired colonies were responding to some conspecific cue that resulted in greater female investment. Bishop *et al.* (2000) found that allosperm induced vitellogenic egg growth not only in the bryozoan *Celleporella hyalina*, but also in the ascidian *Diplosoma listerianum*. Further, Hughes *et al.* (2002a) found that colonies of *C. hyalina* exposed to allosperm produced significantly more female zooids compared to

non-exposed controls. Hunter and Hughes (1995), however, found that the number of male and female zooids in cultured *C. hyalina* colonies varied with differing food and temperature combinations. It appears that sex allocation in bryozoans is complex, and that allocation can vary in response to numerous environmental signals.

Effect of selfing on embryo production

Compared to colonies cultured in pairs, solitary colonies were found to brood significantly fewer embryos, measured as the percentage of filled brood chambers per colony (Tables 1 and 2). This significant difference could be a consequence of various reproductive barriers minimizing selfing. Pre-zygotic barriers to self-fertilization have not been directly investigated in these animals; if rigid mechanisms do exist, embryos could still be produced *via* automixis. Robertson (1903) suggested that this could occur in the stenolaemate cyclostome *Crisia* spp., but this has not been further investigated. For many species of bryozoans, sperm enter the maternal zooid *via* an opening at the base of the lophophore—a supraneural pore or the intertentacular organ (ITO) depending on the species (Temkin, 1994). This entry site could serve as a barrier to self-fertilization through selective uptake of non-self sperm. Dyrynda and King (1982), however, found spermatozoa in the coelomic cavity of a female zooid on a colony that had been maintained in isolation for several days. Additionally, Temkin (1994) found that in isolated colonies of *M. membranacea*, the ITO allowed spermatozeugmata to enter the coelomic cavity of maternal zooids. These studies suggest that there is little regulation of sperm entry into female zooids, but rather that any conspecific sperm contacting a lophophore is transferred to the maternal coelom. Once inside the maternal zooid, genetically based barriers such as those described for *Ciona intestianilis* (Harada *et al.,* 2008) could prevent self-fertilization. Temkin (1991), however, found that some coelomic oocytes extracted from isolated colonies of *M. membranacea* contained sperm nuclei. It seems likely, therefore, that the significant difference in brooded embryos between treatments documented in my study was a result of post-zygotic inbreeding depression—specifically, increased embryonic abortion. Although it was not quantified due to the large amount of debris from the UFSW and introduced algal cells that would accumulate each day, aborted embryos were routinely found at the base of solitary colonies. Large decreases in numbers of brooded embryos did not necessarily correlate with increased larval output in this treatment. For instance, in 2008 one solitary colony was found to contain 123 brood chambers, 108 (≈87%) of which were filled. Larval releases conducted over the next four days resulted in a total of five larvae released, but the number of brooded embryos decreased from 108 to 37. The majority of these embryos were most likely aborted, and these rates of decrease without increased larval output were not observed in the paired treatment colonies.

Effect of selfing on offspring fitness

Offspring from solitary colonies experienced reduced fitness compared to offspring from colonies in the paired treatment. Solitary colonies released significantly fewer larvae and had significantly fewer individuals initiating and completing metamorphosis (Fig. 2; Table 3). Not only was there reduced larval output from these colonies, but rates of metamorphic initiation and completion were also reduced. For example, in 2009 paired colonies released a total of 646 larvae. Of these, 87% initiated metamorphosis and 95% of these completed metamorphosis. Solitary colonies released a total of 129 larvae; 71% initiated metamorphosis and 65% completed metamorphosis. Hence, not only were there fewer larvae released *via* selfing, but the fitness of these selfed larvae was also significantly compromised. By transplanting metamorphs back to the field in 2009, I was also able to show that for those offspring that were able to complete metamorphosis, selfing resulted in decreased survival and reproductive fitness compared to outcrossed controls (Table 4). These results demonstrate that, although selfing can occur in *B. stolonifera*, there are significant deleterious effects manifested at every stage of growth from embryos to reproductively mature colonies. Further, they suggest that selfing is rare in this population. If selfing were prominent, it would be expected that deleterious alleles would have been purged over time and these animals would be able to self with little to no inbreeding depression (Crnokrak and Barrett, 2002). For instance, Swindell and Bouzat (2006) provided evidence that the purging of deleterious recessive alleles in *Drosophilia melanogster* led to significantly reduced inbreeding depression in certain lineages. The results from my study suggest that the Eel Pond population of *B. stolonifera* is neither selfing nor maintaining a mixed-mating system, but is routinely outcrossing.

As previously discussed, it does not appear that there is strict discrimination between self and non-self sperm by maternal zooids. Hence, how these animals minimize selfing in a natural population remains unclear. One mechanism could be a consequence of the distribution pattern of bryozoans within a given locale (see Ryland, 1973). Bryozoans often form patchy distributions, whereby high numbers of adults are contained within a small spatial scale. Further, bryozoan larvae are known to settle on adult colonies, such that a bryozoan colonial mass could be made up of multiple, genetically distinct individuals. Keough (1989b) found no consistent deleterious effects on growth and survival of *B. neritina* juveniles growing adjacent to mature colonies. It could be that this settling behavior and patchy distribution, without resulting in intra-specific competition, allow for ample opportunities to outcross, as well as minimize the

chances of taking up own-self sperm. Investigations into the genetic relatedness of individuals within these patchy distributions are currently being conducted.

Selfing in natural populations

The results from my experiments suggest that although the Eel Pond population of *B. stolonifera* is capable of selfing, this is not routinely occurring, as evidenced by the significantly reduced fitness of selfed offspring compared to outcrossed controls. If this decreased fitness were due to deleterious alleles, then successful selfing events could rapidly purge these alleles, particularly when selection favors selfing over outcrossing. Long-distance dispersal events can lead to the introduction of small numbers of individuals into a new area, and theoretically a single self-compatible individual could colonize an area following this type of introduction (Baker, 1955). Results from investigations with *Celleporella hyalina* showed a differential ability to self among geographically distinct populations (Hughes *et al*., 2009). These findings suggest that the ability for a population as a whole to self might be traced back to the colonizing individuals. For instance, if the population within a given locale were founded by few individuals, the selection for selfing would be greater and could result in the establishment and subsequent propagation of self-compatible individuals. In my study, solitary colonies were indeed able to reach reproductive maturity, self, and release offspring. Selfed offspring were themselves shown to reach reproductive maturity and release larvae. Therefore, it remains a possibility that under different circumstances, selfing in *B. stolonifera* could lead to the production of viable, self-compatible offspring. Results from this study, however, establish that *B. stolonifera* colonies in Eel Pond are not selfing, but rather are routinely outcrossing, and that any potential selfing events would result in the production of inviable offspring.

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Chapter 3

Larval settlement preference maximizes genetic mixing in an inbreeding population of a simultaneous hermaphrodite (Bryozoa, *Bugula stolonifera*)

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Larval settlement preference maximizes genetic mixing in an inbreeding population of a simultaneous hermaphrodite (*Bugula stolonifera*, Bryozoa)

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Abstract

Conspecific aggregations in terrestrial and aquatic organisms can have a significant effect on an individual's survival, growth and reproductive fitness, particularly if these aggregations are composed of closely related individuals. Such aggregations can form passively, as a consequence of dispersal, or actively, as a consequence of kin recognition. In this study, we investigated the genetic composition of individuals in conspecific aggregations in the simultaneous hermaphroditic marine bryozoan *Bugula stolonifera*. Conspecific larvae routinely metamorphose on adult colonies; the possibility that larvae select or avoid their maternal colony was investigated utilizing 10 newly developed polymorphic microsatellite loci. Adult colonies were collected from Eel Pond, Woods Hole, Massachusetts and inspected for the presence of attached individuals. Adult colonies and their attached individuals were genotyped and compared to assess genetic relatedness within and among these groups relative to the overall genetic variability of the sampling site. Overall, the population of *B. stolonifera* at this site was found to be outside Hardy–Weinberg equilibrium because of significant levels of inbreeding. No significant genetic differentiation, however, was found between any groups, documenting that a group containing an adult colony and its attached individuals had as much genetic variability as was found for the entire sampling site. Parentage-exclusion analyses showed that the vast majority of attached individuals (>93%) could not have derived from the colony on which they were attached. Kinship analyses showed that the majority of attached individuals (≈63%) shared less than a half-sibling relationship. These results suggest that a colony's nearest neighbours are not composed of siblings, and thus, larval settlement preference can maximize outcrossing in this inbreeding population.

Keywords: conspecific aggregations, kinship, larval settlement, outcrossing

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Introduction

It has been observed for some time that the spatial distribution of terrestrial and aquatic organisms can be patchy, whereby high densities of conspecifics occur on a relatively small spatial scale (Allee 1931). Despite many species possessing a larval stage with potentially high dispersal capabilities, these types of conspecific aggregations have been shown to be quite common in benthic marine invertebrates (Meadows & Campbell 1972; Burke 1986; Pawlik 1992). Understanding the formation of such aggregations is an important step to assessing potential ecological implications, including the effects these aggregations have on an individual's survival, growth and reproduction. We sought to examine the genetic structure of conspecific aggregations in the marine bryozoan *Bugula stolonifera*. Bryozoans often form dense aggregations of adults (e.g. Keough & Chernoff 1987) and can be good systems for investigating the potential consequences of this lifestyle.

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A seminal study investigating the formation of conspecific aggregations in the barnacle *Balanus balanoides* showed that larvae preferentially settled adjacent to either previously settled conspecifics or the base plates of conspecifics (Knight-Jones 1953). This work was among the first to demonstrate that larvae are capable of discerning between substrates and thus are active in their choice of settlement site and also that these larvae settle gregariously. Subsequent work investigating similar settlement behaviour in various benthic invertebrates has shown that planktonic larvae respond to numerous cues when choosing settlement site (see review by Pawlik 1992), including surface energy (see review by Callow & Fletcher 1994), surface topography (Walters & Wethey 1996; Walters *et al.* 1997), biofilms (see reviews by Scheltema 1974; Hadfield & Paul 2001), food source (Morse & Morse 1984; Hadfield & Pennington 1990) and conspecifics (Patzkowsky 1988; Jensen 1989; Pearce & Scheibling 1990). The choice of settlement site during the larval phase is of paramount importance, particularly to sessile adult organisms, and these cues are used as indicators of sites ideal for growth and reproduction in later life stages. Consequently, widespread response to these cues and gregarious larval settlement within a species can lead to increased intraspecific competition. It is thought, however, that the benefits of group-living, such as reduced predation, increased foraging, protection from physical stressors and increased reproductive success, outweigh any potential costs incurred from monospecific aggregations (see Boucher *et al.* 1982; Stachowicz 2001). Further, it has been suggested that altruism promoting group-living will be more strongly selected for when groups are composed of closely related individuals (Hamilton 1963, 1964). Indeed, studies examining population genetic structure suggest that aggregations of closely related individuals might be relatively common in marine systems.

Studies examining population genetics in numerous species have found genetic structure on small spatial scales (see review by Jackson 1986), indicating that pockets of closely related individuals can exist within a larger population. Species that brood offspring and release lecithotrophic larvae are characterized as having limited dispersal potential. Hence, kin aggregations can arise simply as a consequence of larval swimming duration. Yund & O'Neil (2000) found genetic differentiation in the ascidian *Botryllus schlosseri* on a scale of several meters, while Calderón *et al.* (2007) found genetic structure in the sponge *Crambe crambe* on a scale of tens of centimetres, with the authors from both studies partly attributing the observed patterns to limited larval dispersal. Additionally, small-scale genetic heterogeneity is thought to occur passively as a result of limited dispersal via asexual processes (see review by Jackson & Coates 1986), as well as potential retention of sibling larvae because of currents (Véliz *et al.* 2006). Alternatively, similar patterns of kin aggregations can form as a consequence of active larval settlement via kin recognition.

Kin recognition is well known for a wide variety of vertebrates and social terrestrial invertebrates (see reviews by Fletcher & Michener 1987; Hepper 1991; Holmes 2004). For marine invertebrates, kin recognition has been primarily investigated in clonal species, usually in studies investigating allorecognition systems that are thought to mediate the outcome of contact between conspecifics (see review by Grosberg 1988). These outcomes can result in aggressive behaviour between the individuals (Francis 1973, 1988; Richardson *et al.* 1979; Ayre 1982; Chornesky 1983), peaceful coexistence at the point of contact (Francis 1973; Ottaway 1978; Bigger 1980), or even fusion, which has been demonstrated in sponges (Wilson 1907; Warburton 1958; Hildemann *et al.* 1979; Ilan & Loya 1990), cnidarians (Duerden 1902; Buss *et al.* 1984; Shenk & Buss 1991), ascidians (Bancroft 1903; Schmidt 1982), and bryozoans (Humphries 1979; Ryland 1979; Chaney 1983; Ishii & Saito 1995; Hughes *et al.* 2004). Although numerous studies have conclusively demonstrated kin recognition in juveniles and adults, few studies have investigated kin recognition by marine invertebrate larvae. Keough (1984) found that sibling larvae of the arborescent bryozoan *Bugula neritina* aggregated, suggesting a kin-recognition mechanism existed in this species. Grosberg & Quinn (1986) also found that sibling larvae of the colonial ascidian *Botryllus schlosseri* aggregated and additionally provided evidence of a link between larval kin recognition and histocompatibility, suggesting that the potential for post-metamorphic colony fusion could be driving the observed aggregation of sibling larvae. Fusion is not known to occur in *Bugula neritina*, but Keough (1984) provided evidence that groups of *B. neritina* colonies could better withstand fish predation compared to isolated colonies. More recently, Hoare *et al.* (1999) showed that larvae of the bryozoan *Celleporella hyalina* settled randomly, neither settling adjacent to or actively avoiding siblings and suggested that this settlement pattern could lead to increased genetic mixing promoting outcrossing.

Our study was undertaken to investigate possible kin aggregations in the arborescent cheilostome bryozoan *Bugula stolonifera* Ryland 1960. *Bugula stolonifera* is cosmopolitan in temperate and tropical waters (Rodgers & Woollacott 2006). Previous work has shown that larvae of this species preferentially settle adjacent to established colonies (Patzkowsky 1988), often forming dense aggregations of adults. Gooch & Schopf (1970) used

enzyme electrophoresis in an early population genetics study to document a lack of genetic structuring within an aggregation encompassing an area of 0.36 m^2. This work was based largely on one polymorphic locus; however, subsequent genetic studies on these aggregations have not been conducted. Additionally, we have observed that larvae will routinely settle and metamorphose on conspecific colonies, with the potential result that a 'colony' of *B. stolonifera* is composed of multiple, genetically distinct individuals. It is possible that larvae of this species have the ability to recognize kin and preferentially settle on or actively avoid their maternal colony. We investigated this possibility using a newly developed microsatellite library. We compared the overall genetic variability within the sample site to that of a colony and its attached individuals. With these comparisons, it was possible to determine whether the individuals attached to a colony were more genetically similar to that colony or to themselves, when compared to the overall genetic variability within the sampling site.

Methods

Microsatellite library development

A microsatellite library was constructed using the protocol developed by Glenn & Schable (2005). Adult colonies were collected in fall 2007 from the Woods Hole Oceonographic Institute, Marine Biological Laboratory (MBL) pier in Eel Pond, Woods Hole, MA. Colonies were fixed in 95% EtOH; then, DNA was extracted using the Qiagen DNEasy Blood and Tissue kit. Two individuals were selected for the microsatellite enrichment and cloning process, which resulted in a total of 384 plasmids. These plasmids were picked and polymerase chain reaction (PCR) amplified with M13 forward and reverse primers, then sequenced with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) on an ABI 3730xl DNA sequencer. Sequences were edited and assembled using Sequencher v. 4.8. Of the 384 sequences, 63 were identified as microsatellites with sufficient flanking regions allowing for primer design. Primer pairs were designed using Primer3 v. 0.4.0 (Rozen & Skaletsky 2000), synthesized (Integrated DNA Technologies, Inc.) and tested for amplification. Fifteen primer pairs were found to amplify consistently. The 5′ end of the forward primer of these pairs was fluorescently labelled (6-FAM™), and PCR conditions were further optimized for genotyping. Polymerase chain reactions were performed in a 12.5 μL volume containing 15–30 ng DNA, 1X PCR buffer, 1.2 mM MgCl, 0.15 mM dNTPs, 0.8 μM of each primer and 0.2 U Ampli*Taq* DNA polymerase. The following thermal cycler programme was used: 94 °C for 4 min, 35 cycles of 94 °C for 30 s, annealing temperature (Table 1) for 30 s, 72 °C for 45 s and 72 °C for 3 min as a final extension step. Polymorphism in these 15 loci was examined in a total of 30 individuals collected from Eel Pond. PCR products were run on an ABI 3730xl DNA sequencer using GeneScan™—500 Rox™ (Applied Biosystems) as the size standard. Results were analysed using PeakScanner Software v1.0 (Applied Biosystems). The number of alleles per locus, observed and expected heterozygosities, and polymorphic information content were calculated using Cervus 3.0 (Kalinowski *et al.* 2007). The presence of null alleles at each locus was investigated using MICRO-CHECKER v. 2.2.3 (van Oosterhout *et al.* 2004). Deviations from Hardy–Weinberg equilibrium (HWE), heterozygote excess or deficiencies and linkage disequilibrium among loci were analysed using GENEPOP 4.0 (Rousset 2008). The sequential Bonferroni correction was utilized to adjust significance levels compensating for multiple comparisons within the same test (Rice 1989).

Genetic variability of attached individuals

Animal collection. Adult colonies were collected from the MBL pier in Eel Pond in summer 2009. To assess overall genetic variation for these animals, 30 colonies sampled from the entire area of the pier (≈15 m^2) were genotyped. Care was taken to ensure that only portions of colonies lacking brood chambers were used. Also, as *B. stolonifera* colonies asexually reproduce *via* root-like projections, only unique genotypes were used for genetic analyses. Additional colonies (3.0–4.5 cm in length) collected from the MBL pier were inspected for the presence of attached individuals (see Fig. 1). A colony and all of the individuals attached to it were then grouped, as were the colonies representing the overall genetic variation, and pairwise comparisons were conducted among all groups. Sampled individuals were fixed, DNA was extracted, and the microsatellite loci were amplified and scored as previously described.

Genetic analyses. Descriptive statistics including the number of alleles per locus, percentage of polymorphic loci and observed and expected heterozygosity were calculated with GDA v. 1.1 (Lewis & Zaykin 2002). Exact tests examining linkage disequilibrium between all pairs of loci and deviation from HWE within each group were conducted in GENEPOP, with Markov chain parameters set to 10 000 dememorizations, 500 batches and 10 000 iterations per batch. For groups outside HWE, estimations of Wright's inbreeding coefficient (F_{IS}) were calculated and tested for significance using GENETIX v. 4.05 (Belkhir *et al.* 1996–2004) set to 10 000 permutations.

Table 1 Accession numbers and characteristics of microsatellite loci developed for *Bugula stolonifera*

GenBank no./Locus	Repeat motif	Primer sequence (5′-3′)	T_a (°C)	No. of alleles	Size (bp)	H_E	H_o	PIC
HM807353/Bug.stol1	(ATG)5	F: CAATTGGGAATCAGGGTCTG R: TGCTACACGTTGTTTGATCG	54	2	243–246	0.452	0.400	0.346
HM807354/Bug.stol2	(CT)7	F: TGTGATCATTCCATCTACTTATCTG R: ATAATGAACAGGAGTCAGTC	62	3	199–203	0.406	0.200*	0.332
HM807355/Bug.stol3	(GTCA)16	F: ATGCAGCCTACCTGACAGAC R: CTCACTCTCCTGCACTCTCTAGGAT	62	9	177–232	0.821	0.500*	0.781
HM807356/Bug.stol4	(CTA)12 CTG (CTA)4 (TTA)4	F: CATCCAGCAGAGAAACTG R: CTCTTTCAGCTTAGGAGAAC	60	11	239–286	0.492	0.233*	0.458
HM807357/Bug.stol5	(CAGT)5 (CAAT)2 (CAGT)4 CAAT (CAGT)4 TAGT CAAT (CAGT)8	F: ATCATCAAATGCAGCCAGTCGG R: GCAAATTCCACTGTCCTTGGAG	60	6	216–249	0.740	0.367*	0.703
HM807358/Bug.stol6	(CTA)15 (CTGCTACTA)6	F: CTGTAGCCTACCGCTGATATTCTC R: TGCTCTACCATGCACATCAACC	60	8	239–292	0.758	0.267*	0.716
HM807359/Bug.stol7	(ACTG)17	F: CGTCTAACTCCTCTTACTTTG R: ACAGTTAGCATACATTGGTG	54	10	181–276	0.864	0.567*	0.833
HM807360/Bug.stol8	(TGAC)7 TGGCTAC (TGAC)7	F: AGAGCAAATCTGCATGTC R: CTATGGGATTCCGTATAGAG	60	3	188–200	0.540	0.467	0.475
HM807361/Bug.stol9	(TCTG)7	F: AGCTCACATGGAGACACAGC R: CATTGCAATATTTGCGGACA	54	4	280–294	0.510	0.200*	0.437
HM807362/Bug.stoll0	(ACT)11	F: ATTGCACACCCACATATC R: AGCTCAGAGGCTTATCATAG	54	10	168–210	0.828	0.500*	0.793

The 5′ end of each forward primer was labelled with 6-FAM™. Primer annealing temperatures (T_a) were optimized for each primer pair. Number of alleles and size range in base pairs (bp) are indicated for each locus. Expected heterozygosity (H_E), observed heterozygosity (H_o) and polymorphic information content (PIC) were calculated using Cervus 3.0. Significant deviations from expected Hardy–Weinberg equilibrium indicated with *(GENEPOP 4.0, $P < 0.005$). $N = 30$ for all loci.

Homogeneity among the seven groups was initially investigated using the exact test for genotypic differentiation in GENEPOP. This test analyses the distribution of diploid genotypes in all pairs and assumes genotypes are distributed equally (Raymond & Rousset 1995). Settings for the Markov chain reaction were as previously

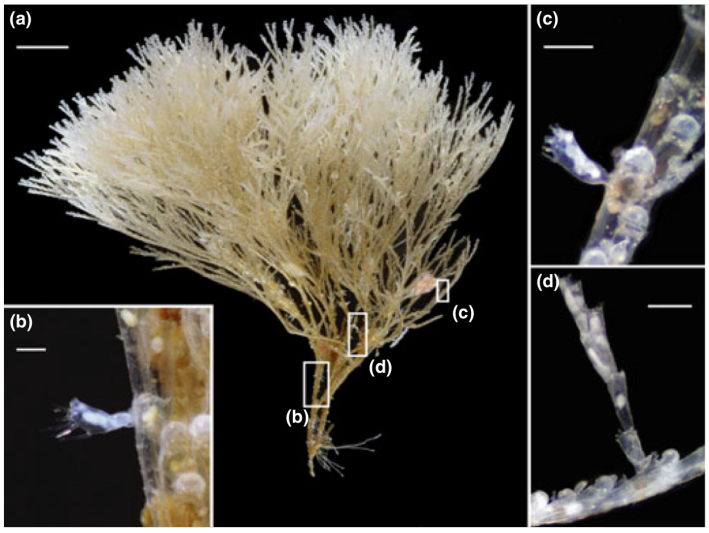

Fig. 1 Bugula stolonifera colony (a) with attached ancestrulae (b, c) and juvenile (d). Ancestrulae from this species develop spines that are not maintained in budded zooids, making attached juveniles distinctive relative to the rest of the adult colony. Scale bars = 5 mm (a), 350 μm (b, c) and 500 μm (d).

described. Genotypic differentiation among all pairs of groups was also analysed by estimating Wright's F_{ST} in Arlequin ver. 3.5.1.2 (Excoffier & Lischer 2010). These values were tested for significance using 10 000 permutations. Significance levels were adjusted using the sequential Bonferroni correction for all analyses conducting multiple comparisons under one test (Rice 1989).

Kinship analyses. In addition to testing for genetic differentiation among groups, a potential parent–offspring relationship between the adult colony and all of its attached individuals was examined. Larvae of *B. stolonifera* are short-lived with a low dispersal potential. It is possible that these larvae might preferentially attach to or actively avoid attaching to their maternal colony. To investigate this, genotypes of attached individuals were inspected for unique alleles not shared by the colony on which they were attached. Hence, at a particular locus, if an attached individual in either the homozygous or heterozygous state did not possess a shared allele with the putative maternal colony, it could be disqualified from having originated from that colony. One problem with this type of parentage exclusion is that the presence of null alleles can lead to erroneous exclusion of the putative parent (Dakin & Avise 2004). Therefore, a more conservative parentage analysis was also conducted incorporating the possibility of null alleles at all loci. Here, if both the adult colony and attached individual were homozygous at a particular locus, the potential parent–offspring relationship could not be disqualified as they might share a null allele. If the adult colony (or attached individual) was heterozygous at a particular locus, however, the parent–offspring relationship could be disqualified if the attached individual (or adult colony) did not possess either allele at that locus, regardless of its allelic state.

The minimum number of parents required to account for the genotypic variation of the attached individuals within a colony was also estimated using the programme COLONY v 2.0 (Jones & Wang 2009). This programme utilizes the maximum likelihood method to estimate the number of parents for a particular brood, without needing prior knowledge of the parental genotype(s). Previous work examining the effectiveness of COLONY found that one disadvantage of this programme was that occasionally the results were overestimations of the actual minimum value (Sefc & Koblmüller 2009). By manually setting observed alleles to very low frequencies, however, it increases the likelihood within the programme that a shared allele represents a shared parent, and the minimum number of parents needed to account for the observed allelic variability can be estimated (Sefc & Koblmüller 2009). The allele frequency for all observed alleles was set to 0.001, while an artificial allele not found in any group was given a high frequency, making the total allele frequencies at a particular locus equal one. This programme was also used to conduct a likelihood analysis of the relationship of each pair of attached individuals within a group, either full-siblings, half-siblings or unrelated. For these analyses, the allele frequencies representing all of the genotyped individuals, irrespective of designated groups, were used. Analysis parameters were set as full-likelihood, with the mating system set as male and female polygamy with inbreeding. Results from this programme will specify a sibling relationship for any pair of individuals provided the resulting likelihood probability is at least 0.001.

Results

Microsatellite library development

Ten of the 15 microsatellite loci tested were found to be polymorphic, with the number of alleles ranging from 2 to 11 and polymorphic information content ranging from 0.332 to 0.833 (Table 1). Expected and observed levels of heterozygosity ranged from 0.406 to 0.864 and from 0.200 to 0.567, respectively. Significant deviations from HWE were found for eight of the 10 loci, resulting from heterozygote deficiencies ($P < 0.005$). Results from MICRO-CHECKER analyses suggested null alleles were present at all loci outside HWE. The lack of individuals being homozygous for any null allele (Appendix SI, Supporting information), however, suggests deviations from HWE stemmed from other causes. No linkage disequilibrium was found in any pairwise comparison among loci.

Genetic variability of attached individuals

Descriptive statistics. For the overall genetic variability of *Bugula stolonifera* growing on the MBL pier in 2009 ($\approx$15 m^2), 27 of the 30 individuals sampled had unique genotypes. An additional six colonies (area = 9.0–21.0 cm^2/colony) were dissected, and 94 attached individuals (n = 13–18/colony) were sampled (Fig. 1), resulting in a total of 127 individuals divided into seven groups used for genetic analyses (Appendix SII, Supporting information). The average number of alleles per locus ranged from 5.5 to 6.9 for each group, and all loci were found to be polymorphic (Table 2). There was no evidence of linkage disequilibrium for any pairs of loci. All groups, including that representing overall genetic variability for the collecting site, were found to deviate significantly from HWE ($P < 0.0001$). Expected heterozygosity ranged from 0.588 to 0.685, while observed heterozygosity ranged from 0.306 to 0.420. Deviations from HWE

Table 2 Summary of genetic diversity of Bugula stolonifera groups from Marine Biological Laboratory pier in Eel Pond, Woods Hole, MA

Group	N	P_L	A	H_E	H_O	F_{IS}
C1	16	1.0	6.2	0.685	0.388	0.443*
C2	18	1.0	5.7	0.588	0.306	0.487*
C3	14	1.0	5.5	0.621	0.401	0.364*
C4	18	1.0	6.7	0.672	0.391	0.425*
C5	19	1.0	6.2	0.652	0.359	0.456*
C6	15	1.0	5.6	0.633	0.420	0.345*
Pier	27	1.0	6.9	0.681	0.375	0.454*

Each group of C1 through C6 is composed of an adult colony and all of the individuals attached to it, while the group Pier is composed of sampled individuals representing overall genetic diversity of the collection site. Number of individuals genotyped (N), percentage of polymorphic loci (P_L), average number of alleles per locus (A), expected (H_E) and observed (H_O) heterozygosity calculated in GDA. Wright's inbreeding coefficient (F_{IS}) calculated in GENETIX v. 4.05. Significant deviations of F_{IS} from zero denoted by *($P < 0.0001$).

were found in all loci across all populations ($P < 0.0001$), suggesting that null alleles were not responsible for this heterozygote deficiency. Inbreeding coefficients for all groups ranged from 0.345 to 0.487, and all were found to be significantly different than zero (Table 2).

Genetic differentiation. There was no evidence for genetic differentiation among any pairs of groups after sequential Bonferonni correction. Exact tests for genotypic differentiation found no significant difference found between any pair of groups ($\alpha = 0.05$), with an average test statistic of 24.59 ± 7.48 (mean ± 1 SD). Tests estimating Wright's F_{ST} yielded an average value of 0.0177 ± 0.0084, with none of the pairwise comparisons significantly different than zero after 10 000 permutations.

Kinship analyses. A parentage-exclusion analysis was conducted by examining the genotypes of attached individuals for unique alleles not shared by the colony on which they were attached. Results showed that the vast majority of the attached individuals could not have originated from the colony on which they were sampled, regardless of the presence of null alleles. Of the 94 individuals collected from the six colonies, 93 (98.9%) contained at least one unique allele that excluded it from having originated from the colony on which it was attached (Appendix SII, Supporting information). When allowing for null alleles at all loci, 88 of 94 (93.6%) attached individuals could be disqualified. Additionally, the majority of attached individuals within a group shared less than a half-sibling relationship with one another. Analyses examining these relationships showed that, on average, only 4.94% ± 2.08 of all pairs of individuals were estimated to be full-siblings, while 32.46% ± 15.70 were estimated to be half-siblings (Table 3). Finally, the average estimated minimum number of parents needed to account for the genetic variability of attached individuals within a colony was found to be 12.0 ± 1.67.

Discussion

Aggregations of closely related individuals have been documented in numerous marine species (see review by Jackson 1986). These types of aggregations can form passively *via* limited dispersal potential, retention of sibling larvae by current patterns and asexual processes, or actively *via* kin recognition (e.g. Keough 1984; Grosberg & Quinn 1986). In this study, we investigated potential kin aggregations in the marine bryozoan *Bugula stolonifera*. Results from our study suggest aggregations of related individuals commonly occur in this species, as evidenced by lack of HWE and significant inbreeding coefficients for all groups. Conversely, we document that a group composed of a colony and its attached individuals had as much genetic variability as was found for the entire sampling site. We further show that larvae do not preferentially attach to their maternal colony. Hence, any potential kin recognition capability of these larvae would lead to maximizing genetic mixing within inbred populations.

Genotypic differentiation

Modes of dispersal have long been known to effect genetic structure and population distribution (Jackson

Table 3 Number of attached individuals per group, likelihood estimation of the percentage of pairs of individuals having a full-sib (FS) or half-sib (HS) relationship and likelihood estimation of the minimum number of parents needed to explain the observed genotypic variability (COLONY v 2.0)

Group	Attached individuals	FS pairs (%)	HS pairs (%)	Minimum parents
C1	15	6.67	28. 57	12
C2	17	1.47	18.38	14
C3	13	6.41	32.05	10
C4	17	5.88	30.88	13
C5	18	5.88	22.22	13
C6	14	3.30	62.64	10

Total number of pairwise comparisons for C1 through C6 is 105, 136, 78, 136, 153 and 91, respectively. Sibling relationships were included based on a minimum likelihood probability of 0.001.

1986; Jackson & Coates 1986; Palumbi 2003). For species with lecithotrophic larvae, recent evidence suggests that genetic structure can occur on surprisingly small scales (e.g. Yund & O'Neil 2000; Calderón *et al.* 2007). The phylum Bryozoa is dominated by species with larvae that possess limited dispersal. For instance, larvae of *B. stolonifera* can become competent to metamorphose within one hour after release and have been shown to have high rates of metamorphic initiation within this time (Woollacott *et al.* 1989; Wendt & Woollacott 1999). Previous work has shown that extended larval swimming can result in decreased survival and post-metamorphic fitness for species with short-lived larvae (Woollacott *et al.* 1989; Wendt 1996, 1998). To avoid these costs, it might be expected that these larvae will settle soon after release, resulting in aggregations of related individuals. The genetic signature of the subsequent population would be expected to be outside HWE, as was found for our sampled population (Table 2). Similar deviations from HWE, however, could result if populations within Eel Pond were isolated from receiving novel genes or recruits from populations located outside Eel Pond. Eel Pond is a relatively closed body of water with but a single opening to Vineyard Sound, and it might be expected that over time, the population within Eel Pond could become increasingly inbred. A study we conducted concurrently with the present study, however, documented that a population of *B. stolonifera* located in Hadley Harbor, an open body of water that is free to mix with surrounding areas, was outside HWE with an inbreeding coefficient significantly different than zero (unpublished observations). Further, significant genetic differentiation existed between populations separated by only 120 m within Eel Pond. Therefore, a combination of geographic isolation and limited larval dispersal could be responsible for the observed deviations from HWE in the Eel Pond population.

One potential consequence of these two factors might be the formation of patches of closely related individuals (full- or half-sibs) within the sampling site. It might be expected that the attached individuals on a particular colony would have come from a limited gene pool. The resulting genetic variability among the attached individuals would be significantly reduced compared to the overall genetic variability of the site and might show genetic differentiation compared to other groups of attached individuals. We found no significant genotypic differentiation among any of the groups sampled in this study. Hence, a group containing an adult colony and all of its attached individuals occupying an area of up to 21 cm^2 had as much genetic variability as was observed for the whole sampling site, which had an area of approximately 15 m^2.

Estimations of the minimum number of parents needed to account for the genetic variability of attached individuals within a group and the results from the kinship analyses also support high mixing within the site (Table 3). On average, ≈16 attached individuals were genotyped within each group. Results from the estimated minimum number of parents showed an average of 12 individuals were needed to account for the observed genetic variability of these attached individuals. Additionally, for five of the six groups investigated, 60–80% of the possible pairwise comparisons between attached individuals had less than a half-sibling relationship. Only a few studies have examined potential fine-scale genetic structure in bryozoans. Hoare *et al.* (1999) examined four microsatellite loci and found no genetic differentiation between populations of the encrusting cheilostome bryozoan *Celleporella hyalina* separated by up to 8 km, although Goldson *et al.* (2001) documented genetic differentiation between samples of the same species separated by as little as 10 m utilizing the RAPD technique. More recently, Pemberton *et al.* (2007) examined five microsatellite loci in the cyclostome bryozoan *Crisia denticulata* and found that the greatest pairwise genetic similarity existed between individuals sampled at the smallest distance (5 cm), while genetic differentiation occurred between patches of animals separated by 4–8 m. These authors suggested that restricted gene flow existed within patches of these animals, possibly as a result of limited dispersal of sperm and/or larvae. In contrast, our results document high amounts of genetic mixing within the sampling site (≈15 m^2) and that the fine-scale genetic structure found in other species (e.g. Yund & O'Neil 2000; Calderón *et al.* 2007; Pemberton *et al.* 2007) is not found in the Eel Pond population of *B. stolonifera*.

Kin aggregations

Our investigations into the genetic relatedness of a colony and its attached individuals showed that the vast majority of attached individuals could not have been released from the colony on which they were attached. This pattern of settlement could have arisen through larval kin recognition, where larvae discern between their maternal colonies and less closely related individuals, or simply as a consequence of larval dispersal. In general, marine invertebrate larvae have an obligatory dispersal period before metamorphic competence is acquired, which can last on the order of minutes to months depending on the species (Pechenik 1990). Larvae of *B. stolonifera* can become competent to metamorphose within 1 h after release and have been shown to have high rates of metamorphic initiation within this time (Woollacott *et al.* 1989; Wendt & Woollacott 1999).

This short time to metamorphic competence, however, could still be sufficient in allowing the newly released larva to leave the proximity of its maternal colony, decreasing the likelihood of settling on or near it.

Ecological implications

Results from our study document that the sampled population of *B. stolonifera* is outside HWE with an inbreeding coefficient significantly different than zero. Despite this, there appears to be a relatively high degree of mixing within the sampling site. One potential benefit of maximizing mixing within an inbred population could be to minimize the deleterious effects associated with inbreeding. Recent work investigating selfing, the most severe form of inbreeding, in *B. stolonifera* documented significant deleterious effects from selfing (Johnson 2010). Although selfing was possible in this species, selfed offspring had significantly decreased survival compared to outcrossed controls, and those that did survive were unable to successfully reproduce. Potential inbreeding depression stemming from matings between siblings has not been investigated in this species, although Hoare & Hughes (2001) documented significant inbreeding depression in offspring from full- and half-sib matings of the encrusting bryozoan *Celleporella hyalina*. Bryozoans are spermcasters (Bishop & Pemberton 2006); sperm are released to the water column and then taken up and utilized by female zooids for internal fertilization (Temkin 1996, and references therein). The formation of dense aggregations of non-siblings could prevent inbreeding depression by minimizing the chances of selfing, or utilizing sperm from siblings. Indeed, the observed pattern of the adult colonies and their attached individuals sampled in this study suggests that just such aggregations routinely form. It seems probable that this pattern should extend to conspecifics immediately adjacent to these colonies as well. Hence, a colony's nearest neighbours are probably not composed of siblings, but rather more distantly related individuals, serving to minimize inbreeding depression in this otherwise inbred population.

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CHJ and RMW are interested in the physiology, ecology and population structure of marine invertebrates, with particular emphasis on the larval biology of colonial organisms. This work is part of the dissertation research of CHJ, a Ph.D. candidate at Harvard University.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Results from genotyping individuals for microsatellite library development.

Appendix S2 Results from genotyping investigating the genetic relationship of a colony and its attached individuals (A.I.).

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Supporting information for

Larval settlement preference maximizes genetic mixing in an inbreeding population of a simultaneous hermaphrodite (*Bugula stolonifera*, Bryozoa)

by C. H. Johnson and R. M. Woollacott

Molecular Ecology (2010) **19**: 5511-5520

Appendix S1. Results from genotyping individuals for microsatellite library development.

Ind	Bug.stol1		Bug.stol2		Bug.stol3		Bug.stol4		Bug.stol5		Bug.stol6		Bug.stol7		Bug.stol8		Bug.stol9		Bug.stol10	
1	001	001	002	002	008	008	001	001	001	001	001	001	009	009	003	003	004	004	003	003
2	002	002	002	002	007	008	002	002	004	004	005	005	001	009	003	003	003	003	003	008
3	001	002	002	002	006	006	002	002	005	005	005	005	002	008	001	002	003	003	004	004
4	001	002	002	002	006	006	009	009	004	004	001	003	004	009	002	002	003	003	006	006
5	002	002	002	002	004	007	001	002	004	004	004	005	009	009	002	003	002	003	009	009
6	001	002	003	003	007	009	001	003	002	002	001	001	001	002	003	003	004	004	007	008
7	001	002	002	002	008	008	001	003	004	004	001	001	001	001	001	001	004	004	004	004
8	002	002	002	003	002	002	002	008	001	004	004	004	003	010	002	003	003	004	003	003
9	002	002	001	002	009	009	001	001	004	004	005	005	001	005	003	003	001	004	010	010
10	002	002	003	003	006	007	001	001	004	004	006	007	001	001	002	003	003	003	003	003
11	001	002	003	003	007	007	001	001	004	004	001	001	001	003	002	003	003	003	004	004
12	001	002	002	002	006	007	001	010	004	004	001	007	001	003	001	003	001	003	003	003
13	002	002	002	003	002	006	001	002	004	004	005	005	007	009	003	003	003	003	002	002
14	002	002	002	003	002	002	001	001	004	004	001	002	003	008	003	003	003	003	002	008
15	001	002	002	002	008	008	006	006	003	003	001	001	004	004	003	003	003	003	001	004
16	001	002	002	002	006	008	002	003	004	004	004	004	003	004	003	003	003	003	001	003
17	001	002	002	002	006	006	007	007	001	001	002	003	006	006	003	003	003	003	001	004
18	001	001	002	003	005	008	004	004	001	002	002	002	004	004	003	003	003	003	002	004
19	001	002	002	002	007	008	001	001	004	004	003	003	001	001	003	003	003	003	004	009
20	001	001	002	002	006	006	001	001	004	004	001	001	002	002	001	003	003	003	001	002
21	002	002	002	002	002	002	001	001	001	004	001	001	004	007	002	003	003	003	003	005
22	002	002	002	002	007	009	001	011	004	004	001	001	001	006	001	003	003	003	003	003
23	002	002	003	003	007	007	002	002	004	004	002	002	009	009	003	003	003	004	003	004
24	001	002	002	002	002	003	003	005	001	004	003	003	001	003	003	003	004	004	002	009
25	002	002	002	002	007	007	002	002	004	005	002	002	007	007	001	003	004	004	001	001
26	002	002	002	002	007	007	001	001	004	004	002	003	007	007	001	001	003	003	001	001
27	002	002	002	002	006	008	001	001	004	004	001	001	002	008	001	003	004	004	003	004
28	002	002	002	003	003	007	008	008	004	006	001	008	001	001	001	003	003	004	003	004
29	001	002	003	003	007	008	001	004	004	004	001	001	002	003	001	002	003	003	003	003
30	001	001	002	002	001	002	001	010	004	005	001	001	001	003	002	003	001	001	003	005

Appendix S2. Results from genotyping investigating the genetic relationship of a colony and its attached individuals (A.I.).

	Bug.stol1	Bug.stol2	Bug.stol3	Bug.stol4	Bug.stol5	Bug.stol6	Bug.stol7	Bug.stol8	Bug.stol9	Bug.stol10
Colony 1	002 002	002 002	009 009	003 019	005 007	001 001	001 001	001 003	007 007	005 009
A.I. 1*†	002 002	003 003	003 003	003 003	005 005	006 006	006 010	003 003	007 007	004 005
A.I. 2*†	002 002	002 003	002 002	011 011	002 005	008 008	011 014	001 003	007 008	002 002
A.I. 3*†	002 002	002 003	003 003	002 011	005 006	004 010	010 010	001 003	007 007	004 010
A.I. 4*†	002 002	001 003	011 011	002 011	004 004	001 003	001 010	003 003	007 007	003 003
A.I. 5*†	002 002	002 003	009 013	003 003	005 005	002 002	001 010	002 003	007 007	003 012
A.I. 6*†	001 001	003 003	003 004	007 007	005 005	002 002	005 014	001 001	007 007	003 004
A.I. 7*†	001 002	003 003	003 003	002 007	005 005	009 009	005 013	002 002	005 005	004 005
A.I. 8*†	002 002	002 002	007 007	016 018	005 005	006 006	006 014	003 003	007 007	004 004
A.I. 9*	001 002	002 002	009 009	004 004	005 005	004 004	013 013	003 003	007 007	005 009
A.I. 10*†	001 002	003 003	003 003	003 007	005 006	009 009	005 005	001 003	007 007	002 004
A.I. 11*†	001 002	002 002	002 003	003 011	003 003	012 012	001 006	001 003	006 007	004 004
A.I. 12*†	001 002	001 001	002 002	002 002	001 005	002 010	013 014	002 002	007 007	004 005
A.I. 13*†	002 003	002 002	009 009	010 010	001 005	001 001	006 011	002 003	001 001	010 013
A.I. 14*†	002 002	002 002	002 002	003 003	005 005	010 010	001 001	003 003	007 007	003 003
A.I. 15*†	002 002	002 002	002 002	003 011	005 007	001 001	001 006	002 004	006 006	002 002
Colony 2	002 002	002 003	002 003	003 003	005 007	004 004	006 006	003 003	007 007	004 004
A.I. 1*†	002 002	002 003	006 006	004 004	005 005	004 004	006 006	003 003	007 007	005 005
A.I. 2*†	002 002	002 002	009 009	003 003	005 005	001 001	001 006	001 002	004 008	003 005
A.I. 3*†	001 002	002 003	009 012	007 007	001 005	002 010	006 009	003 003	007 007	004 005
A.I. 4*†	002 002	003 003	006 006	002 002	005 005	004 004	005 011	003 003	007 007	004 005
A.I. 5*†	002 002	002 003	011 013	007 008	005 005	009 009	001 006	002 003	007 007	003 005
A.I. 6*†	002 002	002 002	007 007	003 003	005 005	002 002	005 005	003 003	007 007	002 003
A.I. 7*†	002 002	004 004	009 009	003 014	005 005	004 010	005 005	003 003	007 008	003 003
A.I. 8*†	002 002	002 004	011 014	011 011	005 005	002 004	006 006	002 002	007 007	004 009
A.I. 9*†	002 002	002 002	007 007	011 011	003 003	001 001	002 002	003 003	005 005	004 005
A.I. 10*†	002 002	002 003	003 003	003 003	001 001	004 004	001 001	003 003	007 007	002 002
A.I. 11	001 002	003 003	002 002	003 011	005 005	004 004	006 013	003 003	007 007	003 004
A.I. 12*†	002 002	002 003	007 007	002 004	005 005	010 010	001 001	001 003	007 007	005 005
A.I. 13*†	002 002	003 003	001 007	003 003	005 005	012 012	005 006	003 003	007 007	003 003
A.I. 14*†	002 002	002 003	007 007	014 014	005 005	001 001	010 013	003 003	007 007	003 004
A.I. 15*†	001 002	002 003	002 002	002 011	005 005	001 001	008 014	003 003	007 007	005 005
A.I. 16*†	002 002	002 002	007 007	003 003	004 005	001 001	005 005	001 002	007 007	009 009
A.I. 17*†	001 002	003 003	002 002	002 003	005 005	011 012	013 013	001 003	007 007	007 007

* denotes attached individuals that could not have originated from the colony on which they were attached
† denotes attached individuals that could not have originated from the colony on which they were attached, while assuming null alleles at all loci

Appendix S2 continued

	Bug.stol1	Bug.stol2	Bug.stol3	Bug.stol4	Bug.stol5	Bug.stol6	Bug.stol7	Bug.stol8	Bug.stol9	Bug.stol10
Colony 3	001 002	002 002	009 011	011 011	005 006	001 001	006 013	003 003	007 007	003 003
A.I. 1*†	002 002	003 003	009 013	002 011	005 005	010 010	001 008	003 003	005 007	002 002
A.I. 2*†	002 002	002 002	- -	011 021	005 005	- -	005 005	001 003	007 007	003 003
A.I. 3*†	001 002	002 002	007 009	019 020	005 005	001 001	001 002	002 003	008 008	002 002
A.I. 4*†	002 002	002 002	002 003	007 007	003 005	004 010	002 003	002 002	007 007	012 012
A.I. 5*†	002 002	002 003	002 007	004 007	005 005	002 002	001 005	003 003	007 007	002 004
A.I. 6*†	002 002	002 003	004 007	002 011	005 005	004 004	005 005	001 002	007 007	003 006
A.I. 7*†	002 002	002 002	007 007	004 004	005 005	001 004	005 005	001 001	007 007	004 004
A.I. 8*†	002 002	003 003	003 003	003 007	005 005	009 009	013 014	002 002	007 007	005 005
A.I. 9*†	002 002	003 004	013 013	002 003	005 005	004 005	005 006	003 003	005 007	009 009
A.I. 10*†	002 002	002 004	014 014	004 004	005 005	002 010	011 015	002 003	007 007	003 004
A.I. 11*†	001 002	002 003	007 007	011 011	005 005	001 004	006 014	003 003	007 007	002 012
A.I. 12*†	002 002	002 002	003 004	004 004	005 005	001 001	011 011	003 003	005 007	003 005
A.I. 13*†	001 002	003 003	009 011	005 005	001 005	005 010	006 014	003 003	007 007	009 009
Colony 4	002 002	003 003	002 003	003 019	005 007	004 004	006 006	003 003	007 007	004 004
A.I. 1*†	001 002	002 003	002 007	020 020	005 005	010 010	001 001	002 003	001 001	004 012
A.I. 2*†	002 002	002 004	014 014	007 011	005 005	001 002	011 015	001 001	007 007	003 007
A.I. 3*†	002 002	003 003	009 009	003 003	005 005	001 004	001 001	003 003	007 007	004 004
A.I. 4*†	002 002	002 004	003 009	003 016	005 005	002 004	006 013	003 003	007 007	003 007
A.I. 5*†	002 002	002 002	002 002	003 003	002 005	- -	005 005	001 003	- -	002 003
A.I. 6*†	002 002	002 003	009 009	007 007	005 005	005 010	001 002	002 003	007 007	004 005
A.I. 7*†	002 002	003 003	002 003	002 003	006 006	004 005	001 006	002 003	005 007	004 009
A.I. 8*†	001 002	002 003	006 006	003 005	003 008	011 012	001 001	003 003	007 007	003 010
A.I. 9*†	002 002	002 002	007 009	011 016	005 005	002 010	006 007	002 002	007 007	004 004
A.I. 10*†	002 002	002 002	002 002	016 016	005 006	001 004	005 005	001 002	005 005	004 011
A.I. 11*†	002 002	002 002	009 009	002 002	001 006	007 010	002 005	002 002	007 007	012 012
A.I. 12*†	002 002	003 003	009 009	005 005	004 004	001 005	002 002	001 001	001 001	002 002
A.I. 13*†	002 002	002 002	003 007	003 006	004 004	005 005	005 008	003 003	005 007	001 004
A.I. 14*†	001 002	002 003	003 003	007 015	005 005	001 010	001 001	001 004	002 007	004 004
A.I. 15*†	002 002	003 003	003 003	012 012	005 005	011 012	014 014	003 003	007 007	004 010
A.I. 16*	002 002	003 003	002 002	003 003	005 005	005 005	005 005	002 003	007 007	009 009
A.I. 17*†	002 002	004 004	006 006	- -	005 005	005 010	006 006	001 003	008 008	009 009

* denotes attached individuals that could not have originated from the colony on which they were attached
† denotes attached individuals that could not have originated from the colony on which they were attached, while assuming null alleles at all loci
- denotes failed amplification

Appendix S2 continued

	Bug.stol1	Bug.stol2	Bug.stol3	Bug.stol4	Bug.stol5	Bug.stol6	Bug.stol7	Bug.stol8	Bug.stol9	Bug.stol10
Colony 5	002 002	- -	003 003	017 017	005 007	004 004	005 005	001 003	007 007	004 004
A.I. 1*†	001 002	002 003	009 009	002 003	006 006	001 001	005 005	003 003	007 007	004 004
A.I. 2*†	002 002	002 002	007 007	022 022	005 005	- -	005 005	001 001	005 008	003 003
A.I. 3*†	002 002	002 002	007 007	003 011	005 005	001 010	005 008	002 004	007 007	002 002
A.I. 4*	002 002	002 003	003 007	004 004	005 005	012 012	005 014	001 002	007 007	003 004
A.I. 5*†	002 002	002 002	007 007	002 011	003 003	010 010	006 006	003 003	001 003	003 005
A.I. 6*†	001 002	002 002	009 013	003 003	- -	011 011	001 003	001 002	002 005	004 004
A.I. 7*†	002 002	002 002	007 011	003 011	005 005	001 010	005 005	002 002	007 007	002 002
A.I. 8*†	002 002	003 003	006 013	003 003	002 005	001 001	005 008	003 003	006 007	003 004
A.I. 9*†	002 002	003 003	007 011	001 001	005 007	001 001	001 001	002 002	007 007	004 004
A.I. 10*†	002 002	002 002	007 007	002 007	005 005	- -	001 008	003 003	005 005	002 002
A.I. 11*†	002 002	002 002	003 007	002 007	005 005	002 002	005 005	002 003	005 005	001 002
A.I. 12*	002 002	002 002	003 007	002 002	005 005	002 002	005 008	001 003	005 005	- -
A.I. 13*†	002 002	003 003	009 013	008 011	005 005	001 001	001 006	003 003	007 008	005 011
A.I. 14*†	002 002	002 002	009 011	002 007	005 007	001 004	001 006	003 003	007 007	008 008
A.I. 15*†	003 003	002 003	007 007	011 011	003 003	009 009	003 009	003 003	001 003	005 010
A.I. 16*†	001 001	002 003	009 013	003 014	005 005	001 001	002 008	003 003	007 007	005 010
A.I. 17*†	002 002	002 002	002 013	002 002	005 005	010 010	006 014	002 003	007 007	004 012
A.I. 18*	002 002	003 003	007 007	007 007	005 005	004 004	005 009	003 003	006 007	005 005
Colony 6	002 002	002 002	009 009	003 019	005 005	001 009	001 001	001 003	007 007	005 009
A.I. 1*†	002 002	002 002	011 011	002 002	005 007	001 010	011 013	001 002	007 007	012 012
A.I. 2*†	002 002	002 002	009 009	011 011	005 005	009 012	001 003	002 003	007 007	003 009
A.I. 3*†	002 002	002 002	006 009	011 011	005 005	- -	001 005	001 003	007 007	004 009
A.I. 4*†	002 002	002 004	006 009	002 014	001 005	002 010	001 001	002 003	007 008	005 005
A.I. 5*†	002 002	003 003	007 011	002 013	005 007	001 001	001 003	002 003	007 007	005 005
A.I. 6*†	001 002	002 002	011 013	002 002	005 005	012 012	001 008	003 003	001 007	003 003
A.I. 7*†	002 002	002 002	007 011	003 007	005 005	001 001	005 005	003 003	008 008	004 004
A.I. 8*†	002 002	003 003	005 009	002 016	005 005	012 012	005 013	002 002	007 007	004 004
A.I. 9*†	002 002	002 002	009 011	003 011	005 005	004 006	001 001	002 003	007 008	004 004
A.I. 10*†	002 002	002 002	009 009	008 008	001 006	006 006	006 013	002 002	001 002	004 011
A.I. 11*†	001 002	002 003	009 009	- -	003 003	001 009	005 005	002 002	007 007	003 008
A.I. 12*†	001 001	003 003	002 007	003 003	005 005	004 006	005 005	003 003	002 002	011 012
A.I. 13*†	002 002	002 003	009 013	002 011	005 005	004 004	005 005	003 003	005 007	005 009
A.I. 14*†	002 002	002 002	002 007	011 011	005 006	005 005	005 005	002 003	007 007	003 005

* denotes attached individuals that could not have originated from the colony on which they were attached
† denotes attached individuals that could not have originated from the colony on which they were attached, while assuming null alleles at all loci
- denotes failed amplification

Appendix S2 continued

	Bug.stol1	Bug.stol2	Bug.stol3	Bug.stol4	Bug.stol5	Bug.stol6	Bug.stol7	Bug.stol8	Bug.stol9	Bug.stol10
Pier 1	002 002	003 003	004 013	003 003	005 005	010 010	005 009	002 003	007 007	012 012
Pier 2	002 002	002 003	009 009	004 007	005 005	012 012	001 001	003 003	009 009	008 008
Pier 3	002 002	003 003	009 011	007 007	005 005	012 012	001 002	001 003	007 007	003 003
Pier 4	001 001	002 002	002 011	003 008	005 006	010 010	004 011	003 003	007 007	002 003
Pier 5	001 002	003 003	007 012	002 007	005 007	008 008	005 005	001 001	007 007	002 003
Pier 6	002 002	005 005	013 013	003 011	005 005	001 004	005 014	001 002	007 007	003 008
Pier 7	001 002	004 004	002 011	007 011	003 005	004 006	009 011	003 003	005 007	004 004
Pier 8	002 002	002 002	011 012	016 016	005 005	001 009	001 001	001 003	008 008	002 005
Pier 9	003 003	002 003	002 002	003 003	005 006	012 012	009 009	003 003	007 007	003 003
Pier 10	001 001	003 003	009 009	002 002	005 005	001 001	005 005	002 003	007 007	002 002
Pier 11	002 002	002 003	009 011	007 007	005 005	012 012	001 002	001 003	007 007	004 004
Pier 12	002 002	003 003	007 007	007 007	005 005	010 010	010 010	001 003	007 008	005 005
Pier 13	002 002	002 002	006 006	003 007	005 005	006 009	004 014	003 003	007 007	005 005
Pier 14	002 002	003 003	003 003	023 023	001 005	004 009	005 006	001 003	007 007	002 004
Pier 15	002 002	002 002	013 013	002 002	006 006	004 010	005 005	001 002	008 008	002 002
Pier 16	002 002	002 002	007 007	003 007	- -	010 010	005 011	001 002	007 007	003 003
Pier 17	002 002	001 003	009 013	002 002	005 005	005 005	005 012	002 003	007 007	004 012
Pier 18	001 002	001 004	002 002	002 003	001 005	001 004	005 005	002 002	007 007	004 005
Pier 19	001 001	002 002	007 007	003 011	005 005	008 010	001 001	002 003	005 007	009 011
Pier 20	002 002	002 002	009 009	007 009	005 005	012 012	008 013	002 003	006 006	004 004
Pier 21	002 002	003 003	007 007	007 007	001 005	001 010	005 008	001 002	007 007	005 005
Pier 22	002 002	002 002	013 013	002 002	006 006	004 010	005 014	001 002	008 008	002 003
Pier 23	002 002	002 003	002 002	019 019	005 005	001 001	001 010	003 003	006 006	012 012
Pier 24	002 002	003 003	002 012	019 019	006 006	001 001	001 001	003 003	006 006	002 012
Pier 25	002 002	003 003	003 010	023 023	001 005	004 009	005 006	001 003	007 007	002 004
Pier 26	002 002	002 002	009 013	002 007	005 005	001 010	005 005	003 003	007 007	002 003
Pier 27	001 002	002 002	012 014	016 016	005 005	012 012	001 011	003 003	007 008	005 005

Pier 1 through Pier 27 are sampled individuals representing overall genetic diversity of the collection site (MBL Pier, Eel Pond, Woods Hole, MA)
* denotes attached individuals that could not have originated from the colony on which they were attached
† denotes attached individuals that could not have originated from the colony on which they were attached, while assuming null alleles at all loci
- denotes failed amplification

Chapter 4

Seasonal patterns of population structure in a colonial marine invertebrate (*Bugula stolonifera*, Bryozoa)

(In press in *The Biological Bulletin*)

Abstract

For sessile invertebrates, the degree to which dispersal mechanisms transport individuals away from their natal grounds can have significant ecological implications. Despite possessing larvae with limited dispersal potential, high levels of genetic mixing have been found within conspecific aggregations of the marine bryozoan *Bugula stolonifera*. In this study, we investigated whether this high mixing within aggregations of *B. stolonifera* also resulted in high mixing between aggregations. Adult colonies were collected from five sites within and one site outside of Eel Pond, Woods Hole, Massachusetts in August 2009 and genotyped at 10 microsatellite loci. Significant genotypic differentiation was found between most sites, suggesting limited connectivity across sites, even those separated by only 100 m. This investigation was extended to determine if low levels of genetic mixing throughout the reproductive season could result in increased homogeneity between sites. Four of the five sites in Eel Pond were sampled early, mid-, and late in the reproductive season in 2010, and again in early 2011. Inter- and intra-annual genotypic differentiation were then assessed within and between sites. Results from these analyses document that low levels of mixing could result in increased homogeneity between some aggregations, but that barriers to genetic exchange prevented mixing between most sites. Further, results from inter-annual comparisons within sites suggest that any potential homogeneity achieved throughout the reproductive season will likely be lost by the beginning of the next reproductive season due to the annual cycle of colony die-back and re-growth experienced by *B. stolonifera* colonies in Eel Pond.

Introduction

Larvae are the primary means of dispersal for sessile invertebrates, although other processes can result in the dispersal of post-metamorphic individuals (*e.g.*, fragmentation, rafting, etc.) (*e.g.*, Bruno, 1998; Watts *et al.*, 1998). The degree to which these dispersal mechanisms actually serve to transport individuals away from their natal grounds can have significant ecological implications, including affecting levels of inbreeding within populations and connectivity between populations. It has been widely accepted that larval dispersal distance can be directly affected by the length of the larval planktonic period (*e.g.*, Crisp, 1978; Bohonak, 1999; Shanks *et al.*, 2003), which in turn can be influenced by such factors as reproductive and developmental mode (see Strathman, 1985), egg size (see Thorson, 1950), amount of endogenous reserves (Wendt, 2000), presence of appropriate settlement cues (see Pawlik, 1992), and ability to withstand a protracted larval period (see Pechenik, 2006). Indeed, results from studies examining larval dispersal in either sympatric or closely related species with contrasting reproductive strategies have shown that increased larval duration can result in increased dispersal (reviewed by Bohonak, 1999). Conversely, a growing body of evidence has documented that larvae of some species recruit back to their natal populations, regardless of the length of the larval dispersal period (*e.g.*, Palumbi, 2001; Swearer *et al.*, 2002; Warner and Cowen, 2002).

Knowlton and Keller (1986) were among the first to empirically demonstrate that localized recruitment in long-lived larvae (≈ 1 week for alpheid shrimp) could occur within meters of the birth site. Subsequent studies have demonstrated that this type of settlement by planktotrophic larvae might be widespread (*e.g.*, Ayre and Hughes, 2000;

Jones *et al.*, 2005). Studies such as these have not only shifted conventional wisdom away from equating dispersal potential with realized dispersal, but have also led to studies investigating how environmental factors (*e.g.*, Dupont *et al.*, 2007; Underwood *et al.*, 2009) or larval behavior can result in lower than expected realized dispersal (see Levin, 2006). Todd *et al.* (1998) investigated genetic structure in a nudibranch with a pelagic lecithotrophic larval stage, which although competent to metamorphose within two days of release, could remain planktonic for several weeks. These authors documented significant genetic differentiation in populations that countered expectations of dispersal potential, and suggested that larval behavior was responsible for the observed local recruitment. Hence, it has become apparent that in considering the realized dispersal achieved by long-lived larvae one must consider variables other than simply dispersal potential. Although the paradox of long-lived larvae demonstrating local recruitment appears to be driving the idea that dispersal potential is a poor indicator of dispersal distance, some studies have documented higher than expected larval dispersal in species with low dispersal potential (*e.g.*, Maier *et al.*, 2009).

Miller and Ayre (2008) examined genetic structure in *Goniastrea favulus*, a broadcast spawning coral with negatively buoyant eggs that adhere to the parent colony through fertilization, and whose larvae are also negatively buoyant through development. Despite expectations of reduced dispersal and localized recruitment, similar levels of genetic subdivision were found when compared to another species with buoyant eggs and larvae, suggesting similar dispersal patterns between the two species. Interestingly, the authors mention that for *G. favulus*, the reduced mobility found in the larvae may only last a few days, after which larvae swim actively. It could be that the ability to withstand

an extended larval swimming duration, and cope with the potential deleterious effects of this protracted phase, is the primary factor allowing for greater than expected dispersal by short-lived non-feeding larvae. Conversely, an inability to cope with a protracted larval phase could result in very low dispersal by larvae and genetic structure on surprisingly small scales. Yund and O'Neil (2000) found genetic structure in a brooding colonial ascidian over distances of eight meters, while Calderón *et al.* (2007) found structure in a brooding sponge on a scale of tens of centimeters. The effects of extended larval swimming in these species is not known; however Marshall *et al*. (2003) documented that extending the swimming duration of larvae by 2-3 hours in the colonial ascidian *Diplosoma listerianum* resulted in reduced fitness due to a smaller post-metamorphic size.

Our study was undertaken to investigate genetic structure in the marine bryozoan *Bugula stolonifera*. As with most species in the phylum Bryozoa, *B. stolonifera* is characterized as having low dispersal potential. Metamorphosis is irreversible and adult colonies are sessile. This species releases short-lived non-feeding larvae that are competent to metamorphose within approximately one hour, and will usually commence metamorphosis within approximately four hours (Woollacott *et al.*, 1989; Wendt and Woollacott, 1999). Previous work has documented significant deleterious effects associated with a protracted larval phase, as extended larval swimming results in significant decreases in juvenile survival and growth (Woollacott *et al.*, 1989). Despite this, Johnson and Woollacott (2010) documented high amounts of mixing within conspecific aggregations. For the present study, a suite of 10 microsatellite loci was used to determine if high mixing within an aggregation also resulted in high levels of mixing between aggregations. For *B.*

stolonifera, however, multiple generations occur throughout the reproductive season; the time to reach reproductive maturity is approximately 12 days (Johnson, 2010), while the embryonic brooding period has been observed in the laboratory to last approximately 7 days (unpublished observations). Thus, additional analyses were performed to determine: 1) if genetic differentiation between sites was consistent throughout the reproductive season, resulting in stable micro-geographic structure for these animals, 2) if there was any evidence for genetic mixing that might result in increased homogeneity between sites, and 3) if inter-annual changes in genetic variability existed within sites. For some erect bryozoans, the erect portions of colonies are not perennial. Rather, these portions experience seasonal die-backs during unfavorable conditions (*e.g.*, Keough and Chernoff, 1987). It is thought, however, that root-like projections emanating from these colonies can survive and can subsequently bud new zooids when favorable conditions return (Numakunai, 1960, 1967). By examining inter-annual genotypic variation in Eel Pond, it is possible to determine if this annual cycle of die-off and re-growth can result in a population bottleneck or shift in genetic composition.

Materials and Methods

Animal collection

Approximately 30 adult colonies of *Bugula stolonifera* Ryland 1960 were collected from each of five sites within Eel Pond, Woods Hole, MA in August, 2009 (Fig. 4.1). A more distantly located site (Hadley Harbor) was also sampled for comparison. To ensure adequate sampling, animals were sampled from the entire area ($\approx$ 5–15 m^2) within each site. As these animals brood embryos within the colony, care was taken to

use only portions of colonies lacking brood chambers for genotyping. Results from genetic analyses conducted in 2009 showed significant genotypic differentiation between most sites. These analyses were extended in 2010 and 2011 to investigate potential inter-annual changes in genetic structure within each site in Eel Pond, as well as intra-annual changes in genetic structure within and between the Eel Pond sites. In Eel Pond, *B. stolonifera* colonies are reproductively active from approximately June through November. The Eel Pond sites used in 2009 were sampled early, mid- and late summer in 2010, and then in early summer 2011. As previously mentioned, multiple generations can occur throughout the reproductive season. Hence, sampling discrete cohorts of individuals is not possible. By preferentially sampling small colonies over large, established individuals at each sampling time, however, it was possible to investigate a change in genetic structure over time. Additionally, under-sampling individuals during each collection could result in an inadequate representation of the genetic diversity found within each site. To ensure that potential significant differences within and between sites were not due to our sampling protocol, an additional collection of animals was conducted at EP 5 during the mid-summer 2010 collection.

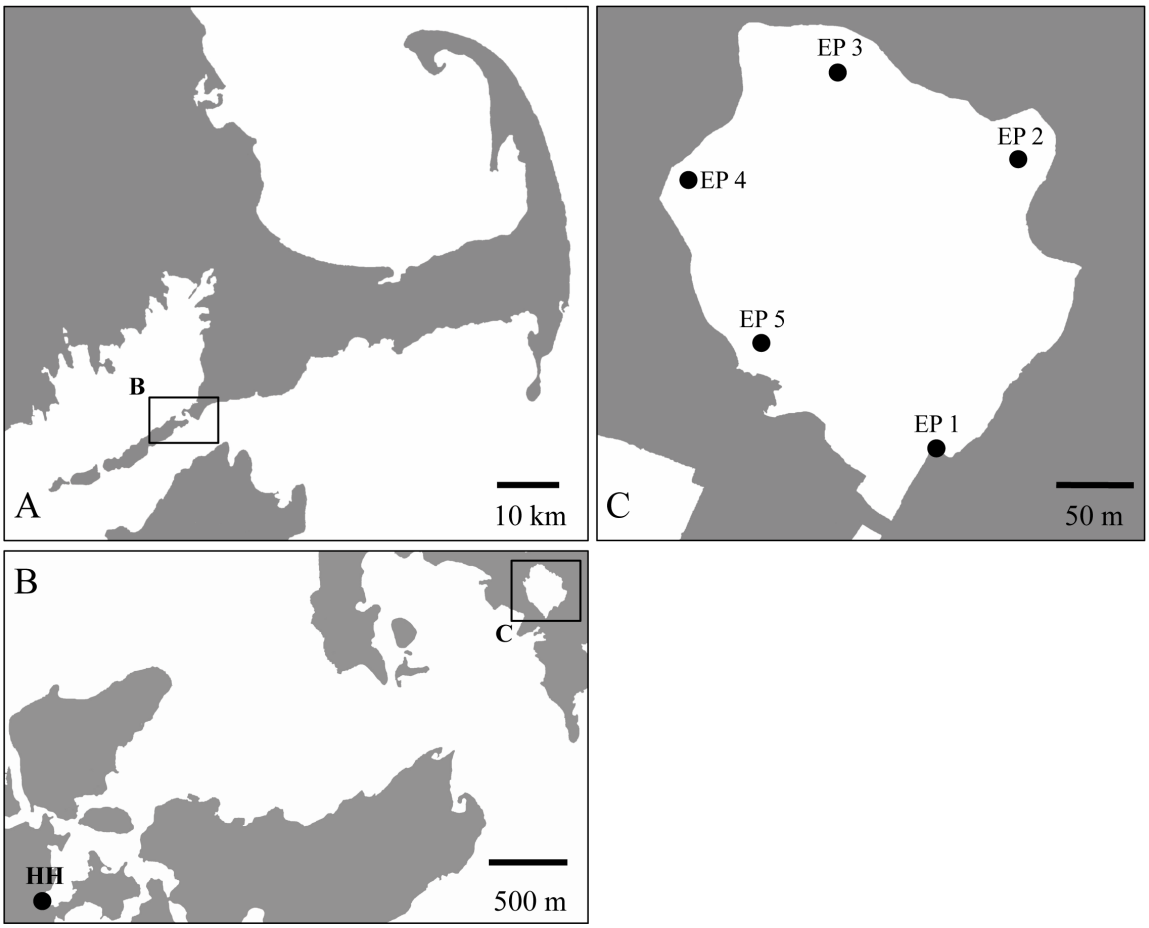

Figure 4.1. Map of SE Massachusetts (A), with insets showing locations of sampling sites outside of (B) and within (C) Eel Pond, Woods Hole. HH – Hadley Harbor, EP – Eel Pond.

Microsatellite amplification

Animals were fixed in 95% EtOH and DNA was extracted using the EZ-10 96 Well Plate Genomic DNA Isolation Kit (Bio Basic Inc.). As *B. stolonifera* colonies brood embryos in the colony, DNA was only extracted from portions of colonies lacking brood chambers. Also, these colonies can asexually reproduce *via* root-like projections. Therefore, only individuals with unique genotypes were used for analyses. Ten microsatellite loci were amplified using published primer pairs (Johnson and Woollacott, 2010). These pairs were divided into three groups based on annealing temperature. The

5′ end of the forward primer in each group was fluorescently labeled with either 6-FAM™, VIC®, NED™, or PET® dye and utilized in a QIAGEN® Multiplex PCR reaction. Reactions were performed in 10 μL volumes containing >20 ng DNA, 1X Multiplex PCR Master Mix, 1X primer mix (0.3 μM each), and 2.75 μL dH_2O. The following thermal cycler program was used: 94°C for 15 min, 30 cycles of 94°C for 30 s, annealing temperature for 90 s, and 72°C for 60 s, followed by a final extension of 72°C for 10 min. PCR products were run on an ABI 3730 xl DNA sequencer using GeneScan™-500 LIZ™ as the size standard. Results were analyzed using PeakScanner Software v1.0 (Applied Biosystems). For loci that failed to amplify during the Multiplex reaction, single locus PCR reactions were performed using the protocol in Johnson and Woollacott (2010).

Data analyses

Descriptive statistics including the number of alleles per locus, percentage of polymorphic loci, and the observed and expected heterozygosity were calculated with GDA v. 1.1 (Lewis and Zaykin, 2002). The presence of null alleles at each locus was investigated using MICRO-CHECKER v. 2.2.3 (van Oosterhout *et al*., 2004). Exact tests examining linkage disequilibrium between all pairs of loci within each year and deviations from Hardy-Weinberg Equilibrium (HWE) within each site were conducted in GENEPOP v. 4.0, with Markov Chain parameters set to 10,000 dememorizations, 500 batches, and 10,000 iterations per batch. Estimations of Wright's inbreeding coefficient (F_{IS}) were calculated and tested for significance using GENETIX v. 4.05 (Belkhir *et al*., 1996-2004) set to 10,000 permutations. The sequential Bonferroni correction was

utilized to adjust significance levels compensating for multiple comparisons within the same test (Rice, 1989).

To estimate the spatial and temporal components of population genetic structure over the duration of the study, a hierarchical analysis of molecular variance (AMOVA) was performed in Arlequin. Genotypic data from each sampled individual were compiled by sampling year, and then grouped according to site. As the Hadley Harbor and EP 2 sites were only sampled in 2009, these data were excluded from this analysis. Additionally, although multiple collections were conducted in 2010, data were compiled into a single sampling period within each site. The locus-by-locus AMOVA for genotypic data in Arlequin partitioned the molecular variance among spatial groups, among collection years within spatial groups, and among samples. Estimated fixation indices were averaged across all loci, and their significance determined using 10,000 permutations. Following this analysis, data were examined for genetic differentiation between samples, and to investigate the potential effects of the seasonal colony die-back within and between sites.

For the 2009 data, genetic differences between the Eel Pond and Hadley Harbor collection sites were initially investigated using the exact test for genotypic differentiation in GENEPOP. This test analyzes the distribution of diploid genotypes in all pairs, and assumes genotypes are distributed equally (Raymond and Rousset, 1995). Settings for the Markov Chain reaction were as previously described. Genotypic differentiation between all pairs of sites was also analyzed by calculating pairwise F_{ST} values in Arlequin ver. 3.5.1.2 (Excoffier and Lischer, 2010). These values were tested for significance using 10,000 permutations. The relationship between genetic and

geographic distance was analyzed by a Mantel test performed in Genetic Analysis in Excel (GenAlEx) v. 6.0 (Peakall and Smouse, 2006). The genetic distance matrix used in this analysis was based on mean pairwise population differences, while the geographic distance matrix was based on decimal latitude and longitude coordinates for each collection site. Significance was determined based on 9,999 permutations.

To investigate potential genetic differentiation resulting from the annual cycle of colony die-back and re-growth, the 2009 data and the early summer 2010 data were subjected to the exact test for genotypic differentiation in GENEPOP and the estimation of pairwise F_{ST} values in Arlequin. To determine if the observed pattern was consistent across multiple years, comparisons were also conducted between the late summer 2010 and early summer 2011 data. Settings for each test were as previously described. Results from these analyses provided evidence for significant differentiation within sites. The early summer 2010 and 2011 data were then investigated for a potential population bottleneck using the program BOTTLENECK v. 1.2.02 (Cornuet and Luikart, 1996). BOTTLENECK utilizes allele frequency data to detect recent reductions in effective population size by comparing observed genetic heterozygosity to the expected equilibrium heterozygosity. Because the allelic diversity decreases faster than genetic heterozygosity during a bottleneck event, populations that have experienced a recent bottleneck should possess a higher than expected heterozygosity, based on the observed allelic diversity. Data were analyzed using the two-phase model of mutation (TPM). The TPM is recommended for microsatellite data as it is a compromise to the stepwise mutation model and the infinite allele model, allowing for mostly stepwise mutations but incorporating a small percentage of multi-step mutations. Estimations from the program

were based on 10,000 iterations. In addition to the use of BOTTLENECK, potential changes in allelic diversity following the annual die-back were examined. If numerous individuals were unable to survive the overwintering process, it might be expected that rare alleles would drop out from each population, resulting in an overall decrease of allelic diversity. The allelic diversity averaged over all loci was calculated for each site at each sampling time in Arlequin.

Prior to examining intra-annual changes in genotypic differentiation within and between sites for the 2010 data, an initial comparison was conducted between the two collections at EP 5 in the mid-summer collection time to ensure that significant differentiation was not due to some artifact of our sampling protocol. These data were subjected to the tests for genotypic differentiation in GENEPOP and Arlequin as previously described. As results documented no significant difference, these data were combined for all subsequent analyses, and the differences between sites within each sampling time were determined. Additionally, intra-annual changes within each site were investigated by comparing the early summer to the mid-summer collection period, and then comparing the mid-summer to the late summer collections. These data were analyzed in GENEPOP and Arlequin as previously described. As Eel Pond *B. stolonifera* colonies experienced widespread colony die-back between the mid- and late summer collections, the late summer data were also analyzed for a potential population bottleneck resulting from this colony die-back. These data were subjected to the tests in BOTTLENECK and examined for changes in allelic diversity as previously described.

Results

Descriptive statistics and results from AMOVA

Throughout the duration of the study, 648 individuals were genotyped from five sites within and one site outside of Eel Pond, Woods Hole, MA (Fig. 4.1). The average number of alleles per locus ranged from 6.6 to 8.7 for each site, and all loci were found to be polymorphic (Table 4.1). All sites in each year were found to deviate significantly from Hardy-Weinberg equilibrium (HWE) ($p < 0.0001$). Expected heterozygosity ranged from 0.641 to 0.707, while observed heterozygosity ranged from 0.354 to 0.557. Results from MICRO-CHECKER analyses suggested null alleles were present at 6 out of 10 loci due to heterozygote deficiencies at these loci. The extremely low occurrence of null allele homozygosity across all loci (≈ 0.17%) (Appendix 4.1), however, suggests that deviations from HWE stemmed from other causes. Inbreeding coefficients ranged from 0.205 to 0.478 and all were found to be significantly different than zero (Table 4.1). There was no evidence for linkage disequilibrium for any pairs of loci after sequential Bonferroni correction, with the exception of *Bug.stol4* and *Bug.stol6* in the EP 1 site in 2009 and *Bug.stol3* and *Bug.stol7* in the EP 4 site in 2011 (see Johnson and Woollacott, 2010 for locus descriptions).

The hierarchical AMOVA investigating overall genetic structure documented both a highly significant spatial component and a highly significant temporal component to the observed genetic variability found throughout the duration of the study (Table 4.2).

Table 4.1. Summary of genetic diversity for *Bugula stolonifera* colonies sampled at Eel Pond (EP) and Hadley Harbor (HH) collection sites in 2009 and early, mid- and late summer 2010. The site labeled EP 5 mid-2 was intended for comparison to EP 5 mid-1 to ensure that our sampling technique was adequately representing the genetic variability of each site at the time of collection. Number of individuals genotyped (N), percentage of polymorphic loci (P_L), average number of alleles per locus (A), expected (H_E) and observed (H_O) heterozygosity calculated in GDA. Wright's inbreeding coefficient (F_{IS}) calculated in GENETIX V. 4.05. Significant deviations of F_{IS} from zero denoted by * (P<0.0001).

	Site	N	P_L	A	H_E	H_O	F_{IS}
2009	EP 1	29	1.0	7.6	0.670	0.483	0.283*
	EP 2	30	1.0	8.0	0.698	0.545	0.222*
	EP 3	19	1.0	6.6	0.700	0.432	0.390*
	EP 4	30	1.0	7.7	0.701	0.492	0.302*
	EP 5	27	1.0	6.9	0.682	0.378	0.451*
	HH	30	1.0	7.9	0.707	0.480	0.324*
2010	EP 1 early	30	1.0	7.7	0.690	0.363	0.478*
	EP 1 mid	29	1.0	7.8	0.685	0.383	0.446*
	EP 1 late	28	1.0	7.6	0.670	0.482	0.284*
	EP 3 early	28	1.0	7.9	0.701	0.439	0.378*
	EP 3 mid	30	1.0	8.7	0.687	0.470	0.320*
	EP 3 late	28	1.0	8.0	0.700	0.527	0.250*
	EP 4 early	30	1.0	8.0	0.683	0.422	0.387*
	EP 4 mid	30	1.0	8.1	0.699	0.383	0.456*
	EP 4 late	30	1.0	8.3	0.686	0.531	0.230*
	EP 5 early	30	1.0	7.3	0.641	0.450	0.301*
	EP 5 mid-1	29	1.0	7.3	0.665	0.421	0.371*
	EP 5 mid-2	29	1.0	7.6	0.654	0.354	0.464*
	EP 5 late	28	1.0	7.6	0.676	0.468	0.311*
2011	EP 1	23	1.0	7.0	0.697	0.557	0.205*
	EP 3	25	1.0	8.0	0.703	0.540	0.235*
	EP 4	26	1.0	8.2	0.696	0.542	0.226*
	EP 5	30	1.0	7.8	0.675	0.510	0.245*

Table 4.2. Results from hierarchical AMOVA investigating the spatial and temporal components of genetic structure over the duration of the study. Because the Hadley Harbor and EP 2 sites were only sampled in 2009, these sites were not included in this analysis. Data were compiled by sampling year, and then grouped according to sampling site. Degrees of freedom were calculated for each locus and are thus not included. Variance components, percentage of variation, and fixation indices were estimated for each locus, and then averaged across all loci. Significance of fixation indices is based on 10,000 permutations.

Source of variation	Sum of squares	Variance components	Percentage of variation	Fixation indices	*P*
Among sites	75.593	0.05538 Va	1.57	F_{CT}= 0.0157	<0.0001
Among years within sites	51.808	0.03981 Vb	1.13	F_{SC}= 0.0115	<0.0001
Among samples	3993.322	3.43665 Vc	97.31	F_{ST}= 0.0270	<0.0001

Table 4.3. Matrix of pairwise F_{ST} values (population genetic differentiation, Arlequin ver. 3.5) below diagonal and results from exact tests for genotypic differentiation (GENEPOP 4.0) above diagonal for Eel Pond (EP) and Hadley Harbor (HH) sites collected in 2009. Bolded values indicate significance ($\alpha = 0.05$).

Site	EP 1	EP 2	EP 3	EP 4	E.P. 5	HH
EP 1		**40.46**	**42.50**	**79.51**	27.30	**71.22**
EP 2	**0.020**		30.02	**104.10**	**40.64**	**78.55**
EP 3	**0.035**	0.013		**69.86**	**40.98**	**69.75**
EP 4	**0.040**	**0.046**	**0.034**		**106.19**	∞
EP 5	**0.026**	**0.017**	**0.024**	**0.046**		**74.05**
HH	**0.043**	**0.040**	**0.047**	**0.052**	**0.051**	

Genotypic differentiation 2009

Pairwise comparisons examining genetic differentiation between sampling sites found significant differentiation between most sites, even those separated by as few as 100 m (e.g. EP 4 and EP 5) (Table 4.3). For the site outside of Eel Pond, significant differentiation was found between the Hadley Harbor collection site and all other sites in Eel Pond ($p \leq 0.0001$ for all tests). Within Eel Pond, results from the exact test for genotypic differentiation conducted in GENEPOP showed significant differences in all comparisons with the exception of EP 2 and EP 3 ($p = 0.070$) and EP 1 and EP 5 ($p = 0.127$) (Table 4.3). Likewise, most pairwise F_{ST} values calculated in Arlequin were found to be significantly different from zero, with only the comparison between EP 2 and EP 3 not significant ($p = 0.133$). No significant relationship was found among comparisons of genetic and geographic distances across all sites ($R^2 = 0.138$, $p = 0.324$).

Animal collection 2010-2011

Collection sites within Eel Pond were monitored bi-weekly beginning in April 2010 for initial colony re-growth. The site designated EP 2 (Fig. 4.1), a floating dock, was no longer located within Eel Pond and could not be used for the 2010 analyses. Colonies of *Bugula stolonifera* appeared in Eel Pond in late-May, and by mid-June each site contained sufficient biomass to allow for collection. For the early summer time period, the site designated EP 5 was sampled on June 4, 2010, while the sites designated as EP 1, EP 3, and EP 4 were sampled on June 17, 2010. These sites were sampled again on July 27, 2010 for the mid-summer time period (40 d from end of previous collection). By this time, *B. stolonifera* abundance was such that colonies carpeted the sides of

floating docks and piers where they occurred. For the late summer time period, sampling sites were inspected on September 6, 2010 (41 d from previous collection). At this time, however, all sites lacked sufficient biomass for collection and it was evident that there had been widespread colony die-back within Eel Pond. Sites were monitored over the next several weeks for colony re-growth, and multiple sampling days were required for each site. Collections were conducted on September 13, September 17, and October 4, 2010 to garner enough genetically distinct individuals necessary for genetic analyses.

Collection sites within Eel Pond were again monitored beginning in April 2011 for colony re-growth. *B. stolonifiera* colonies appeared in Eel Pond in mid-June, but growth was delayed relative to the previous year. The sites designated EP 4 and EP 5 were sampled on June 29, 2011; however, EP 1 and EP 3 did not have sufficient biomass present to allow for collecting until mid-July. These sites were sampled on July 12, 2011.

Genotypic differentiation 2010-2011

An initial comparison of genetic differentiation was conducted on the EP 5 mid-summer 1 and mid-summer 2 collections to test if potential genetic differences were due to some artifact of our sampling protocol. No significant difference in genotypic differentiation was found in either GENEPOP ($p = 0.773$) or Arlequin ($p = 0.666$), suggesting that our sampling protocol adequately represented the genetic variability found at that site (Table 4.4). The genotypic data for these two mid-summer collections were then combined and used for all other comparisons.

Table 4.4. Results from pairwise F_{ST} calculations and exact tests for genotypic differentiation for changes in genetic diversity within collection sites in Eel Pond, Woods Hole, MA. The negative control was conducted to ensure that an artifact of sampling of each site was not responsible for significant differences between sampling periods within sites. Inter-annual comparisons were conducted between groups of animals collected from each site in August 2009 and early summer 2010, and between late summer 2010 and early summer 2011. The late summer collection period in 2010 spanned 3 weeks (9/13/2010-10/4/2010) due to widespread *Bugula stolonifera* colony die-off after the mid-summer collection period, possibly resulting from hurricane activity and increased freshwater influx to Eel Pond. Bolded values indicate significance ($\alpha = 0.05$).

	Site	F_{ST}	Genotypic Differentiation
	Negative control		
	EP 5 mid-1 & -2	0.005	15.07
	Inter-annual comparisons		
2009 - 2010	EP 1	0.015	**36.22**
	EP 3	**0.030**	**48.62**
	EP 4	0.011	**43.85**
	EP 5	**0.017**	**45.75**
2010 - 2011	EP 1	**0.032**	**46.72**
	EP 3	0.013	**36.90**
	EP 4	**0.021**	**44.05**
	EP 5	0.006	28.21
	Intra-annual comparisons		
Early - mid	EP 1	0.005	20.32
	EP 3	0.012	29.97
	EP 4	0.009	26.58
	EP 5	0.011	**32.04**
Mid - late	EP 1	**0.019**	**40.45**
	EP 3	0.011	**44.18**
	EP 4	0.015	**38.29**
	EP 5	0.011	**37.12**

Investigations examining inter-annual genotypic differentiation within sites found that significant differentiation can exist between years for some sites, and that this pattern was consistent across multiple years (Table 4.4). Results from the program BOTTLENECK suggest that these significant changes were not due to a population bottleneck. All loci for all sampling sites were shown to fit the TPM model in 2010 ($p \geq 0.348$) and in 2011($p \geq 0.246$), documenting that none of the sampled sites possessed the excess in genetic heterozygosity expected from a recent bottleneck event. Additionally, no substantial decrease in allelic diversity was observed in any site (Fig. 4.2).

For potential intra-annual genotypic differentiation within each site, comparisons found no significant difference between the early and mid-summer collection times for the majority of comparisons (Table 4.4). In contrast, the mid- to late summer tests showed evidence of differentiation within sites. Results from GENEPOP showed significant genotypic differentiation for each site, while the F_{ST} value for EP 1 calculated in Arlequin was found to be significantly different from zero ($p = 0.025$) (Table 4.4). Similar to the inter-annual genotypic differentiation within each site, the genetic differences documented between the mid- and late summer collection periods do not appear to be a result of a population bottleneck, as all loci for all sampling sites were shown to fit the TPM model (BOTTLENECK: $p \geq 0.461$) and there was no observable decrease in the allelic diversity for any site (Fig. 4.2).

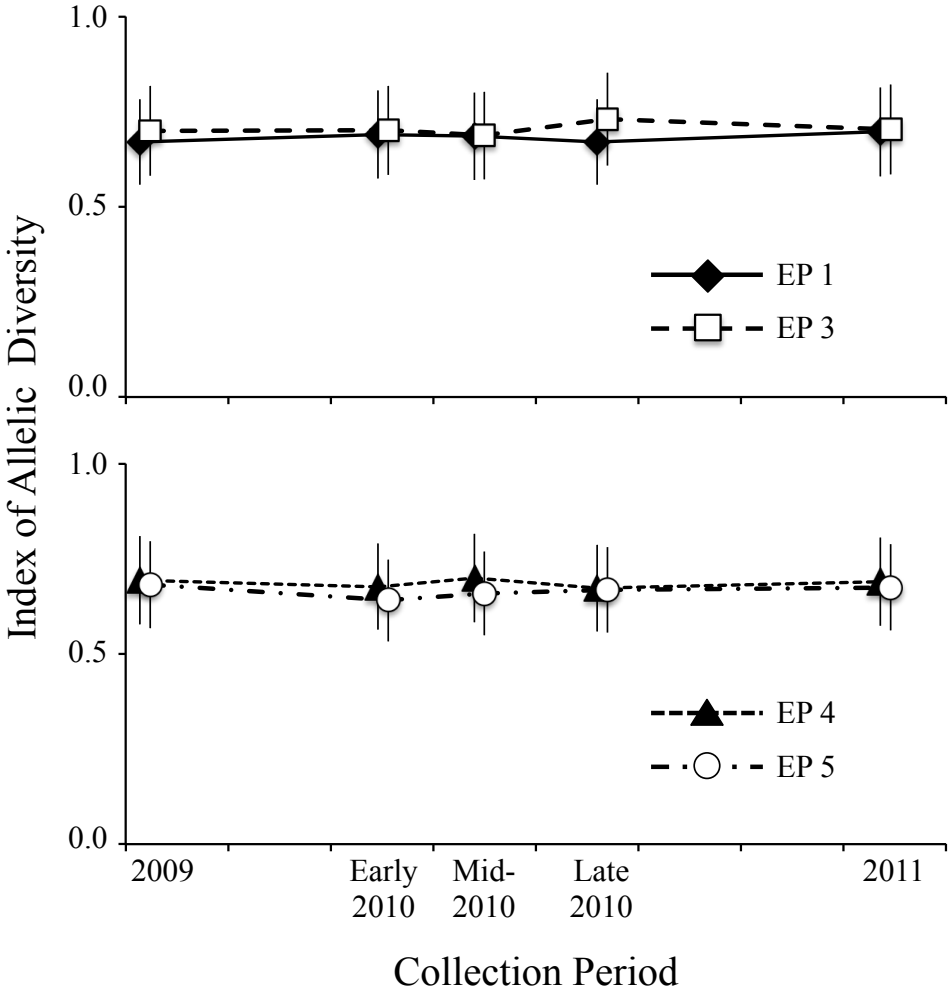

Figure 4.2. Allelic diversity averaged over all loci for each site at each sampling time. Data points are offset and sites separated for ease of viewing. The lack of an observable decrease in allelic diversity for any site throughout the duration of the study suggests that the die-back events experienced by *B. stolonifera* in Eel Pond do not result in a substantial decrease in population size. Bars = 1 S.E.

As in the 2009 comparison, differentiation was found between all sites for the early 2010 collection. An overall calculation of F_{ST} for Eel Pond was 0.034, which was shown to be significantly different from zero ($p < 0.0001$). Results from GENEPOP and Arlequin showed significant differences in all pairwise comparisons (Table 4.5). To examine a change in genetic structure between collection sites throughout the reproductive season, newly settled individuals were preferentially selected over established colonies during the mid-summer collection period. The overall F_{ST} for Eel Pond was found to decrease to a value of 0.020 ($p = 0.001$), documenting continued heterogeneity within Eel Pond. Results from pairwise comparisons did show decreased genotypic differentiation between some sites, but results from the majority of comparisons continued to be significant (Table 4.5). The collection sites designated EP 1 and EP 3 were no longer genetically differentiated, with non-significant results found from analyses conducted in both GENEPOP ($p = 0.258$) and Arlequin ($p = 0.141$). Any trend toward homogeneity did not persist, however, through the late summer collection period. The overall F_{ST} for Eel Pond at this time was found to be 0.024 ($p < 0.0001$), and results from GENEPOP and Arlequin documented continued significant genotypic differentiation in most pairwise comparisons (Table 4.5). For this collection time, the only non-significant comparison was the pairwise F_{ST} calculated between EP 1 and EP 3 ($p = 0.115$).

Table 4.5. Matrix of pairwise F_{ST} values below diagonal and results from exact tests for genotypic differentiation above diagonal for samples conducted in Eel Pond in early, mid- and late summer 2010. Early summer animals were collected in June 2010, while mid-summer animals were collected in July 2010. The duration of the late summer collection was from September 13 to October 4, 2010 due to widespread colony die-off after the mid-summer collection period. Bolded values indicate significance ($\alpha = 0.05$).

Early summer 2010

	EP 1	EP 3	EP 4	EP 5
EP 1		**38.34**	**70.60**	**41.01**
EP 3	**0.019**		**88.92**	**76.64**
EP 4	**0.036**	**0.049**		∞
EP 5	**0.024**	**0.055**	**0.060**	

Mid-summer 2010

	EP 1	EP 3	EP 4	EP 5
EP 1		23.62	∞	**32.83**
EP 3	0.012		**61.65**	**47.84**
EP 4	**0.043**	**0.030**		∞
EP 5	0.013	**0.014**	**0.044**	

Late summer 2010

	EP 1	EP 3	EP 4	EP 5
EP 1		**49.62**	**81.35**	**50.01**
EP 3	0.012		**65.10**	**61.95**
EP 4	**0.038**	**0.030**		**82.95**
EP 5	**0.026**	**0.017**	**0.045**	

Discussion

Previous work examining fine-scale genetic structure in the marine bryozoan *Bugula stolonifera* documented that high levels of mixing occurred within conspecific aggregations (Johnson and Woollacott, 2010). A group of individuals occupying an area of up to 21 cm^2 possessed as much genetic variability as was found for an entire sampling site (area ≈ 15 m^2). In the present study, we examined whether this greater than expected mixing within an aggregation also resulted in increased levels of genetic mixing among aggregations within Eel Pond. Results from collections conducted in 2009 documented significant genotypic differentiation between most comparisons, suggesting that minimum genetic exchange existed between sites. This investigation was extended to 2010 and 2011, whereby potential changes in genetic heterogeneity within and between sampling sites throughout the reproductive season were examined. Results from these analyses documented that some mixing could occur between sites, but that this mixing was not ubiquitous within Eel Pond. Indeed, sites separated by as little as 100 m showed no evidence of interbreeding, suggesting that significant barriers to genetic exchange can exist on small spatial scales. Analyses of temporal genetic structure documented that significant differentiation can occur inter-annually within sites, most likely due to the presumably random survival or differential growth of individuals during the annual die-back experienced by these animals. Taken together these results suggest that regardless of any low probability dispersal events that might lead to increased homogeneity between sites in Eel Pond, the overwintering strategy by these animals will likely lead to increased differentiation at the beginning of the next reproductive season.

Genetic variability within sampling sites

Modes of dispersal have long been known to affect population distribution and connectivity (Jackson, 1986; Grosberg and Cunningham, 2001; Palumbi, 2004). For sessile organisms, the absence of a long dispersal phase could result in the accumulation of closely related individuals on a small spatial scale. Although a few bryozoans have planktonic larval development, the vast majority of species brood embryos within the colony and release short-lived larvae with limited dispersal potential (see Zimmer and Woollacott, 1977). As it has also been documented that extended swimming by non-feeding larvae can result in decreased survival and post-metamorphic fitness (Woollacott *et al.*, 1989; Wendt, 1996, 1998), it might be expected that these larvae will settle soon after release. The resulting genetic signature deriving from this settlement pattern would be expected to deviate significantly from HWE, as was found in this study. All sampling sites were found to deviate significantly from HWE in 2009, 2010 and 2011 due to a deficiency in heterozygotes (Table 4.1). Further, all sites were found to have high inbreeding coefficients that were significantly different from zero. These data suggest that limited larval dispersal can result in the clumping of closely related kin, increasing the potential for inbreeding in these conspecific aggregations. Alternatively, similar deviations from HWE could result in populations that were isolated from receiving novel genes or recruits for long periods of time. Eel Pond is a relatively closed body of water with a single opening to Vineyard Sound (Fig. 4.1), and it might be expected that over time the population within Eel Pond could become increasingly inbred. It is unclear which mechanism is responsible for the observed deviations from HWE in our sampled sites, or if the two processes worked in concert with one another. That the population

sampled in neighboring Hadley Harbor, an open body of water that is free to mix with surrounding areas, was also outside of HWE suggests that limited larval dispersal alone could result in a population deviating significantly from HWE (Table 4.1).

Genotypic differentiation between sites

In addition to resulting in the formation of conspecific aggregations, limited larval dispersal has also been tightly coupled with genetic structure (*e.g.*, Todd *et al*., 1998, Watts and Thorpe, 2006). Indeed, studies have documented that this structure can occur on small spatial scales for species with short-lived larvae (*e.g*., Yund and O'Neil, 2000, Calderón *et al.*, 2007). In our study, results from analyses conducted in 2009 appear to support this assertion. Genotypic differentiation was found in most pairwise comparisons, even between sampling sites separated by only 100 m (*e.g.*, EP 4 and EP 5) (Table 4.3). By examining intra-annual changes in genetic structure among sites in 2010, we were able to show that although some mixing was possible, sites within Eel Pond were not freely interbreeding. For instance, the site designated EP 4 showed no evidence of mixing with any other site throughout the duration of the study (Table 4.5), and when coupled with the high levels of genotypic differentiation suggests this sight was isolated from other sites in Eel Pond. Importantly, the negative control conducted during the mid-summer 2010 collection documents that the observed pattern was not due to an artifact of sampling (Table 4.4). Rather, these results suggest meaningful barriers to genetic exchange can exist on small spatial scales for these animals.

Although these Eel Pond sites do not appear to be freely interbreeding, there was some evidence that low levels of mixing were occurring between some sites. By the mid-

summer collection period, EP 1 and EP 3 were no longer significantly differentiated, and comparisons of these two sites with EP 5 showed decreased values in results from analyses conducted in Arlequin and GENEPOP (Table 4.5). How this mixing occurred remains unclear. As the reproductive season progressed, an increasing number of colonies would become reproductively mature, resulting in an increase in larval output. It seems likely that this increase in larvae would also increase the likelihood of low-probability long-distance larval exchange between sites. Alternatively, this pattern of increased mixing could also have resulted from two other possibilities: long distance sperm transfer or anthropogenically mediated dispersal.

Although fertilization is internal in bryozoans, sperm are released to the water column, a reproductive strategy that is relatively widespread among invertebrate taxa (Bishop and Pemberton, 2006). Animals that use this strategy are not only thought to have long-lived sperm, but also that these organisms can utilize sperm at very dilute concentrations. The longevity of sperm released by *Bugula* spp. has not been investigated, but sperm longevity has been reported for other bryozoans. Manríquez et al. (2001) documented a half-life of 1.2 hr for sperm from the bryozoan *Celleporella hyalina*. Also, Yund and McCartney (1994) provided evidence of long distance fertilization while conducting field-based mating assays with *C. hyalina*, as well as the colonial ascidian *Botryllus schlosseri*. Finally, Temkin (1994) reported that spermatozeugmata released from the bryozoan *Membranipora membranacea* experienced periods of quiescence while in the water column and only commenced strong undulating movements when contacting tentacles of a lophophore. It remains possible that the observed mixing documented in our study resulted not only from larval dispersal, but also

from long distance sperm dispersal, whereby sperm were released at one site and passively transported among other sites.

The second possibility is that the increased homogeneity documented between these sites occurred as a consequence of anthropogenically mediated dispersal. Eel pond is a heavily trafficked area with abundant watercraft maintained throughout the pond. Colonies of *B. stolonifera* routinely grow on boat hulls and the transport of reproductively mature colonies among the sampling sites is certainly possible. If this were the case, however, it is unclear why the site designated EP 4 would not have shown signs of increased mixing as was evidenced between EP 1 and EP 3 (Table 4.5). Additionally, the site designated EP 3, a permanent floating dock anchored within Eel Pond, is a non-functioning dock. Despite this, the role of anthropogenically mediated dispersal cannot be discounted completely, and it remains uncertain how the increased homogeneity between EP 1 and EP 3 occurred.

Any increase in homogeneity among sites observed in the mid-summer collection period of 2010 was not continued through to the late summer collection period (Table 4.5), most likely due to the widespread colony die-back and re-growth observed after the mid-summer collection. For many invertebrates, fluctuations in environmental conditions have been shown to adversely affect adult and offspring survival. For instance, adult mortality due to decreased salinity from heavy rainfall was reported for the ascidians *Ascidia nigra* (Goodbody, 1962) and *Corella willmeriana* (Lambert, 1968). In fact, decreased salinity from heavy rain, flooding, or ice melt has been implicated in the mass mortality of numerous shallow marine invertebrate taxa (*e.g.*, Goodbody, 1961 and references therein). Eel Pond did receive elevated levels of rainfall in late August

and early September that could have resulted in the decline in adult biomass first observed on September 6. The Woods Hole region received approximately 50 mm of rain from August 23 through August 26, and an additional 70 mm of rain associated with Hurricane Earl on September 4 (http://water.weather.gov/precip/). Similar to the seasonal die-back experienced in the winter by *B. stolonifera* (see below), results from analyses seem to suggest that this mid-season die-back did not culminate in a population bottleneck. It could be, however, that because these animals experience a perpetual cycle of bottleneck and population expansion due to the annual die-back, the number of individuals surviving a mass mortality event would have to be minuscule for a bottleneck to be detectable. However, there was no observable decrease in the index of allelic diversity for any site following a die-back event (Fig. 4.2), further suggesting that these events do not culminate in the massive die-off of individuals. Regardless, the presumably random survival of individuals during the mid-season die-back or the differential growth of individuals following the event not only resulted in significant genotypic differentiation within each site between the mid- and late summer collection periods (Table 4.4), but also in increased levels of genotypic differentiation among sampling sites in the late summer collection, relative to those levels found in the mid-summer collection (Table 4.5).

The patterns of genetic structure found throughout this study are also indicative of the problem of conducting population genetic surveys using only a single sampling period, as was conducted in this study in 2009. For species that have short generation times (*e.g.*, Dybern, 1965; Wendt, 1996; Burton, 1997; Massaro and Rocha, 2008), intra-annual changes in population genetic structure are possible. Hence, multiple collections

need to be conducted over time to fully understand the population dynamics of these types of species.

Implications of seasonal colony die-back

While the reproductive season for *B. stolonifera* (≈ June-Oct.) in Eel Pond results in high densities of adults and thick mats of colonies where the animal occurs, the winter months see a decline in animal abundance, culminating in the absence of the erect portions of colonies at or just below the water line in most areas. How these animals overwinter to re-populate in the summer months remains unclear. One possibility is that a standing stock of animals survives at depth, presumably below each of the sites where this animal was collected. Even though Eel Pond routinely freezes over during the winter months, the colonies found at depth might be able to survive. Eel Pond, however, consists of a sandy bottom, not appropriate for attachment by *B. stolonifera*. Additionally, neither an adult nor any suitable attachment substrate was found on visual inspection of the bottom under the MBL Pier (EP 5) conducted in 2009 (unpublished observations). It seems unlikely, therefore, that the population present in the summer months arise from a standing stock of animals beneath the surface. Alternatively, it has been proposed that bryozoans might over-winter *via* root-like projections (Numakunai, 1967) that emanate from various parts of the adult colony and attach to the substrate to assist in anchoring. These projections can also bud additional zooids that, through asexual reproduction, can develop into a genet. Each winter as the temperature of Eel Pond drops, the erect portion of the colony dies off leaving the root-like projection attached to the substrate. When favorable conditions return, a zooid is budded that

eventually develops into an adult. Results from analyses suggest that this over-wintering strategy does not result in a population bottleneck, nor does it result in a decrease in allelic diversity (Fig. 4.2). Rather, the random survival of individuals following this die-back can result in significant inter-annual genotypic differentiation within collection sites (Table 4.4). Further, this random survival within each site could also result in the high levels of genotypic differentiation observed among sites (Tables 4.3 and 4.5). Hence, any homogeneity between sites resulting from low-probability long-distance genetic mixing during a reproductive season will likely be lost at the beginning of the next season due to the annual cycle of die-back and re-growth.

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Appendix 4.1. Results from genotyping individuals for population genetic analyses. Animals were collected from five sites within Eel Pond (EP) Woods Hole, Ma, and one site outside (Hadley Harbor - HH). In 2010, animals were sampled in early (A), mid- (B), and late (C) summer to examine intra-annual changes in genetic structure. An additional collection was conducted during the mid-summer time period at EP 5 as a negative control (see Methods). * denotes loci with potential null alleles, as suggested by the software package MICRO-CHECKER. - denotes failed amplification (see last row for totals).

Ind	Locus1* ATG5	Locus2 CT7	Locus3* GTCA16	Locus4* CTA12	Locus5 CAGT8	Locus6* CTA15	Locus7 ACTG17	Locus8* TGAC7	Locus9* TCTG7	Locus10 ACT11
2009										
EP 1-01	002 002	002 002	003 012	008 010	006 011	007 007	003 003	001 004	009 009	003 004
EP 1-02	002 002	002 002	013 013	003 004	011 011	014 014	009 016	004 004	011 011	002 004
EP 1-03	002 002	002 002	008 011	003 032	011 011	005 005	003 019	004 004	011 012	001 003
EP 1-04	001 002	002 002	006 011	024 027	010 011	014 014	008 010	003 004	011 012	002 003
EP 1-05	002 002	002 004	002 008	003 003	011 012	014 014	009 018	001 003	011 012	003 008
EP 1-06	001 002	002 002	013 016	007 010	011 011	005 005	003 004	004 005	009 011	004 012
EP 1-07	002 002	002 002	011 011	010 010	012 012	014 014	003 009	003 004	009 011	012 012
EP 1-08	002 002	002 002	011 014	003 003	011 013	014 014	003 010	001 001	011 011	003 008
EP 1-09	002 002	002 003	004 012	003 007	012 012	005 005	009 016	001 003	011 011	004 012
EP 1-10	002 002	002 002	013 014	003 003	004 011	002 014	015 019	003 003	011 011	005 013
EP 1-11	001 001	002 002	011 011	002 004	011 012	002 014	013 016	001 001	012 012	003 005
EP 1-12	002 002	002 003	013 016	003 003	011 011	002 014	011 012	004 004	011 011	005 007
EP 1-13	002 002	002 002	013 014	005 005	011 011	010 010	009 010	001 004	010 010	004 004
EP 1-14	001 002	002 002	003 003	032 032	011 011	005 014	010 010	004 004	009 009	004 005
EP 1-15	002 002	002 002	011 011	004 011	004 011	005 014	003 009	004 004	011 012	002 004
EP 1-16	002 002	003 003	011 016	004 004	004 011	005 005	012 015	001 003	012 012	003 004
EP 1-17	002 002	002 002	011 016	004 024	011 011	016 016	010 018	001 003	009 012	004 005
EP 1-18	001 001	002 002	014 016	002 003	011 011	010 010	018 019	004 004	011 012	003 013
EP 1-19	002 002	002 003	005 005	004 032	011 011	002 010	003 009	004 004	010 010	003 005
EP 1-20	001 002	002 002	003 014	032 032	011 011	005 014	010 010	004 004	009 009	004 005
EP 1-21	002 002	002 002	003 003	008 010	006 011	007 007	003 003	001 004	009 009	004 005
EP 1-22	002 002	002 002	013 013	003 004	011 011	014 014	009 016	004 004	011 011	003 005
EP 1-23	002 002	002 003	011 019	010 010	009 009	010 014	003 003	001 001	011 011	004 004
EP 1-24	002 002	002 002	002 002	003 010	011 011	007 007	003 003	004 004	011 012	004 004
EP 1-25	002 002	002 002	011 011	004 011	004 011	005 014	003 009	004 004	011 012	002 004
EP 1-26	001 001	002 002	004 004	002 002	011 011	010 010	018 019	001 003	012 012	009 013
EP 1-27	001 002	003 003	008 014	016 017	011 012	002 009	010 019	003 004	009 014	003 004
EP 1-28	001 002	002 002	008 011	004 010	011 011	002 014	003 015	004 004	011 012	002 004
EP 1-29	001 001	001 002	011 013	017 017	011 012	009 009	010 010	004 004	011 011	002 002
EP 2-01	002 002	003 003	008 011	003 004	011 011	002 014	003 017	001 004	011 012	003 004
EP 2-02	001 002	002 002	008 011	004 037	011 011	006 014	003 018	001 004	010 011	004 004
EP 2-03	002 002	003 003	008 011	002 022	011 011	016 016	007 015	003 004	009 011	003 008
EP 2-04	002 002	002 002	013 013	007 017	014 014	015 015	016 019	001 004	009 011	004 004
EP 2-05	002 002	003 003	008 008	003 003	- -	002 010	003 003	004 004	011 011	003 008
EP 2-06	002 002	003 003	011 014	004 004	011 013	007 014	003 003	003 004	011 011	003 003
EP 2-07	002 002	002 003	014 016	007 010	011 013	007 007	003 003	004 004	002 002	003 004
EP 2-08	001 002	002 002	008 008	004 011	011 011	007 010	007 017	001 004	011 011	002 014
EP 2-09	001 002	003 003	008 008	003 004	011 011	015 015	009 014	001 004	002 002	003 003
EP 2-10	002 002	002 002	011 016	017 022	011 011	002 012	003 003	001 003	010 010	001 003
EP 2-11	001 002	002 003	003 011	002 003	009 009	014 014	019 019	001 003	011 011	011 011
EP 2-12	002 002	003 003	011 017	004 007	001 011	010 010	003 016	003 004	011 011	004 005
EP 2-13	001 002	002 002	011 011	010 017	011 011	016 016	004 009	001 001	012 012	004 004

Appendix 4.1 continued

Ind	Locus1*	Locus2	Locus3*	Locus4*	Locus5	Locus6*	Locus7	Locus8*	Locus9*	Locus10
EP 2-14	003 003	002 003	011 013	003 003	006 011	002 002	003 010	003 004	011 011	004 004
EP 2-15	001 002	002 002	005 016	003 017	001 011	005 007	003 019	003 004	012 012	003 011
EP 2-16	002 002	002 003	011 016	002 004	011 012	010 014	003 017	003 004	007 011	013 013
EP 2-17	001 002	002 004	013 016	003 004	001 011	005 014	010 019	003 004	007 012	003 009
EP 2-18	002 002	003 003	008 014	003 017	011 012	005 006	018 020	003 004	009 011	003 013
EP 2-19	002 002	001 002	008 008	011 022	004 011	014 014	003 015	001 004	011 011	009 011
EP 2-20	002 002	002 002	011 013	003 003	011 013	010 014	018 019	001 004	004 009	002 005
EP 2-21	001 001	002 004	003 003	003 003	011 011	010 014	003 003	004 004	012 012	004 004
EP 2-22	001 002	002 002	008 012	003 003	004 011	003 007	008 019	003 004	010 010	003 013
EP 2-23	002 002	001 002	003 011	011 022	004 011	014 014	003 003	001 004	011 011	003 011
EP 2-24	002 002	002 002	011 013	004 010	011 011	014 014	012 019	003 004	011 011	005 013
EP 2-25	002 002	003 003	005 016	003 003	001 001	006 007	003 016	004 004	011 011	004 004
EP 2-26	002 002	002 002	006 006	003 004	004 011	014 014	003 003	001 004	011 011	009 009
EP 2-27	002 002	002 002	011 016	004 010	001 011	010 010	003 003	001 004	011 011	004 005
EP 2-28	002 002	001 003	005 008	004 037	001 011	014 015	009 011	004 004	011 012	004 004
EP 2-29	002 002	001 004	003 003	003 003	011 013	016 016	009 009	001 004	011 011	003 009
EP 2-30	001 002	003 003	008 008	005 011	011 012	002 014	003 003	003 004	002 002	005 005
EP 3-01	002 002	003 003	011 011	003 017	009 009	008 016	016 018	001 004	012 012	004 004
EP 3-02	001 002	002 004	011 016	003 007	011 012	002 002	015 018	001 004	011 011	003 004
EP 3-03	002 002	003 003	013 016	007 011	011 012	009 009	004 012	001 003	011 012	013 013
EP 3-04	002 002	002 002	008 013	007 011	011 011	006 006	004 012	001 001	012 012	004 004
EP 3-05	002 002	003 003	011 016	003 005	011 011	016 016	010 018	003 003	011 011	001 003
EP 3-06	002 002	001 002	011 019	007 007	003 011	007 010	004 009	001 004	011 011	003 003
EP 3-07	002 002	003 003	011 019	003 003	011 011	007 007	009 015	003 004	011 012	002 004
EP 3-08	001 001	001 003	011 011	037 037	011 012	005 014	003 003	004 004	012 012	002 003
EP 3-09	002 002	003 003	003 003	004 011	011 011	005 010	012 015	004 004	011 011	004 004
EP 3-10	002 002	002 002	003 011	007 019	011 014	008 008	010 010	004 004	012 012	004 013
EP 3-11	002 002	004 004	011 011	004 004	004 011	003 014	003 015	004 004	009 011	005 005
EP 3-12	001 001	003 004	004 015	003 004	011 011	002 002	003 003	001 004	010 010	004 004
EP 3-13	002 002	003 003	011 011	003 017	009 009	008 008	016 018	001 004	012 012	005 005
EP 3-14	002 002	002 002	008 009	004 011	009 012	005 014	003 003	003 003	011 011	005 005
EP 3-15	002 002	003 003	011 011	003 003	011 011	006 007	019 019	004 004	011 011	004 005
EP 3-16	002 002	001 003	008 008	003 003	011 011	002 010	004 004	004 004	011 011	004 005
EP 3-17	001 002	002 002	011 013	004 007	009 009	002 008	009 018	001 004	009 011	004 009
EP 3-18	002 002	002 002	008 008	004 004	011 011	003 005	003 014	003 004	011 011	007 007
EP 3-19	001 002	001 001	012 017	003 003	011 011	002 014	016 016	001 001	009 012	005 005
EP 4-01	002 002	002 002	005 014	004 011	011 011	005 014	009 018	004 005	005 005	003 004
EP 4-02	002 002	002 003	011 011	037 038	011 011	006 010	009 015	003 003	011 011	003 003
EP 4-03	002 002	002 002	005 013	004 022	011 011	005 007	009 011	004 005	009 009	003 004
EP 4-04	002 002	002 002	011 014	019 037	011 011	002 007	009 012	003 004	007 011	005 005
EP 4-05	002 002	001 001	005 016	005 005	011 011	014 014	017 018	003 004	007 007	003 004
EP 4-06	002 002	001 002	007 016	022 022	011 011	008 010	009 009	004 004	011 012	003 013
EP 4-07	002 002	003 003	006 014	005 010	011 011	005 005	003 017	004 004	012 012	003 004
EP 4-08	002 002	003 003	003 008	022 022	011 011	002 014	010 010	004 004	009 012	003 005
EP 4-09	002 002	002 002	004 005	011 011	004 011	005 014	009 009	003 005	005 011	003 004
EP 4-10	002 002	003 003	011 011	010 022	011 012	005 007	009 010	003 005	009 009	002 006
EP 4-11	002 002	001 002	013 016	004 004	003 011	005 007	017 017	003 004	009 009	002 007
EP 4-12	002 002	005 005	011 013	030 030	011 014	005 005	015 015	003 003	011 011	005 005

Appendix 4.1 continued

Ind	Locus1*	Locus2	Locus3*	Locus4*	Locus5	Locus6*	Locus7	Locus8*	Locus9*	Locus10
EP 4-13	002 002	001 001	013 013	003 004	011 011	006 010	009 019	001 004	016 016	004 004
EP 4-14	002 002	003 003	011 016	003 010	011 014	005 005	003 018	003 005	012 014	004 013
EP 4-15	002 002	003 003	011 011	010 028	011 011	005 008	003 009	004 005	014 014	003 013
EP 4-16	001 002	003 003	011 011	003 003	011 011	002 010	009 010	003 004	009 012	004 005
EP 4-17	001 002	001 003	007 007	- -	011 011	005 007	009 009	001 004	009 012	004 007
EP 4-18	002 002	001 001	013 013	003 007	004 011	006 008	003 003	003 004	016 016	001 003
EP 4-19	002 002	002 003	011 011	003 003	011 011	007 007	005 007	004 004	015 015	003 006
EP 4-20	002 002	002 003	005 011	003 011	011 011	005 014	015 015	001 004	009 009	004 004
EP 4-21	002 002	005 005	011 013	004 010	009 015	007 008	003 011	003 004	012 012	004 012
EP 4-22	001 002	002 002	008 011	010 029	004 011	014 014	003 003	001 003	012 012	003 004
EP 4-23	001 002	001 002	011 013	003 004	011 011	005 005	017 017	004 004	009 012	005 007
EP 4-24	002 002	001 002	007 007	030 030	011 011	005 005	010 010	001 003	011 011	002 004
EP 4-25	002 002	003 003	005 005	004 004	011 011	005 005	003 003	004 004	009 011	004 004
EP 4-26	002 002	003 003	011 011	002 010	008 015	005 005	009 009	003 005	009 015	005 013
EP 4-27	002 002	002 002	011 013	002 002	009 011	007 007	009 009	003 004	016 016	005 005
EP 4-28	001 002	001 002	005 007	030 030	009 011	008 010	009 009	001 003	011 012	002 002
EP 4-29	002 002	001 002	003 005	003 003	011 014	014 014	010 010	003 004	009 011	004 005
EP 4-30	001 001	003 003	006 013	005 005	011 014	010 010	017 017	004 004	009 009	004 005
EP 5-01	002 002	003 003	004 016	004 004	011 011	014 014	009 014	003 004	011 011	013 013
EP 5-02	002 002	002 003	011 011	005 010	011 011	016 016	003 003	004 004	014 014	008 008
EP 5-03	002 002	003 003	011 013	010 010	011 011	016 016	003 004	001 004	011 011	003 003
EP 5-04	001 001	002 002	002 013	004 011	011 012	014 014	008 016	004 004	011 011	002 003
EP 5-05	001 002	003 003	008 014	003 010	011 013	009 009	009 009	001 001	011 011	002 003
EP 5-06	002 002	005 005	016 016	004 017	011 011	002 005	009 019	001 003	011 011	003 008
EP 5-07	001 002	004 004	002 013	010 017	009 011	005 007	014 016	004 004	009 011	004 004
EP 5-08	002 002	002 002	013 014	024 024	011 011	002 010	003 003	001 004	012 012	002 005
EP 5-09	003 003	002 003	002 002	004 004	011 012	016 016	014 014	004 004	011 011	003 003
EP 5-10	001 001	003 003	011 011	003 003	011 011	002 002	009 009	003 004	011 011	002 002
EP 5-11	002 002	002 003	011 013	010 010	011 011	016 016	003 004	001 004	011 011	004 004
EP 5-12	002 002	003 003	008 008	010 010	011 011	014 014	015 015	001 004	011 012	005 005
EP 5-13	002 002	002 002	006 006	004 010	011 011	007 010	008 019	004 004	011 011	005 005
EP 5-14	002 002	003 003	003 003	040 040	004 011	005 010	009 010	001 004	011 011	002 004
EP 5-15	002 002	002 002	016 016	003 003	012 012	005 014	009 009	001 003	012 012	002 002
EP 5-16	002 002	002 002	008 008	004 010	009 011	014 014	009 016	001 003	011 011	003 003
EP 5-17	002 002	001 003	011 016	003 003	011 011	006 006	009 017	003 004	011 011	004 013
EP 5-18	001 002	001 004	002 002	003 004	004 011	002 005	009 009	003 003	011 011	004 005
EP 5-19	001 001	002 002	008 008	004 017	011 011	009 014	003 003	003 004	009 011	009 011
EP 5-20	002 002	002 002	011 011	010 015	011 011	016 016	012 018	003 004	010 010	004 004
EP 5-21	002 002	003 003	008 008	010 010	004 011	002 014	009 012	001 003	011 011	005 005
EP 5-22	002 002	002 002	016 016	003 003	012 012	005 014	009 019	001 003	012 012	002 003
EP 5-23	002 002	002 003	002 002	032 032	011 011	002 002	003 015	004 004	010 010	013 013
EP 5-24	002 002	003 003	002 014	032 032	012 012	002 002	003 003	004 004	010 010	002 013
EP 5-25	002 002	003 003	003 012	040 040	004 011	005 010	009 010	001 004	011 011	002 004
EP 5-26	002 002	002 002	011 016	003 010	011 011	002 014	009 009	004 004	011 011	002 003
EP 5-27	001 002	002 002	014 016	024 024	011 011	016 016	003 016	004 004	011 012	005 005
HH-01	001 002	003 003	013 016	003 003	012 012	007 007	009 012	001 001	012 012	001 001
HH-02	002 002	002 002	002 002	003 031	011 011	003 009	016 017	001 001	014 014	003 014
HH-03	001 002	002 002	003 008	036 037	011 012	003 005	003 003	004 004	011 012	010 010

Appendix 4.1 continued

Ind	Locus1*	Locus2	Locus3*	Locus4*	Locus5	Locus6*	Locus7	Locus8*	Locus9*	Locus10
HH-04	002 002	003 003	003 010	003 003	011 012	005 014	009 009	001 001	012 014	003 013
HH-05	001 001	002 002	011 016	004 024	011 012	007 007	009 012	003 004	011 012	003 010
HH-06	001 002	003 003	013 016	003 003	012 012	007 007	009 012	001 001	012 012	001 001
HH-07	002 002	002 002	013 016	003 010	011 011	005 006	003 019	003 003	007 012	003 003
HH-08	001 002	002 002	004 011	003 010	012 012	009 009	003 008	001 001	012 012	003 003
HH-09	001 002	002 002	005 011	003 010	009 012	002 006	003 003	004 004	012 012	003 003
HH-10	001 002	002 002	011 013	004 004	011 011	002 002	008 020	001 004	010 010	003 003
HH-11	002 002	002 004	013 016	003 010	011 011	006 006	003 019	003 003	007 009	003 006
HH-12	001 002	002 002	006 006	010 010	009 011	003 009	003 020	003 003	012 012	003 003
HH-13	002 002	002 002	013 013	011 032	009 011	005 010	016 026	003 004	011 011	003 003
HH-14	002 002	003 003	008 013	005 023	003 011	012 012	003 012	004 005	011 011	003 003
HH-15	001 002	002 002	011 013	004 004	011 011	002 002	008 020	001 004	010 010	003 003
HH-16	002 002	003 003	003 003	003 023	011 012	016 016	003 016	004 004	009 011	003 003
HH-17	002 002	002 003	005 013	003 003	009 012	003 009	003 015	003 004	011 011	003 003
HH-18	001 002	003 003	011 013	004 010	011 011	002 002	008 009	001 003	011 011	003 003
HH-19	002 002	002 002	011 011	003 010	011 014	005 005	003 020	003 005	010 010	003 003
HH-20	002 002	002 002	013 013	003 004	011 011	003 007	020 020	003 004	007 012	003 013
HH-21	002 002	003 003	003 010	003 003	011 012	005 014	009 009	001 001	013 015	003 013
HH-22	002 002	002 002	003 003	002 002	004 011	003 003	020 026	001 003	012 012	004 004
HH-23	002 002	002 002	008 013	004 004	009 009	010 010	003 010	003 003	005 009	005 005
HH-24	002 002	003 003	016 016	004 012	011 012	007 007	003 003	001 004	009 010	004 005
HH-25	002 002	002 003	003 003	004 011	014 014	005 007	016 020	001 003	012 012	004 004
HH-26	001 002	003 003	013 013	010 010	009 012	005 010	003 019	001 001	009 009	003 004
HH-27	001 002	002 002	011 013	010 010	011 012	015 015	003 003	004 004	009 015	003 013
HH-28	001 002	002 003	011 013	004 004	011 011	010 010	008 021	003 004	010 012	004 013
HH-29	002 002	003 003	003 013	004 004	011 012	010 010	016 019	003 004	009 011	004 005
HH-30	002 002	002 002	008 011	004 004	011 011	003 009	013 026	003 004	011 012	003 004
2010										
Early										
EP 1-01	001 001	002 002	005 005	004 004	011 012	005 005	003 003	004 004	011 011	003 005
EP 1-02	002 002	001 002	011 013	004 011	008 011	014 015	003 003	003 003	011 011	003 004
EP 1-03	002 002	001 001	003 003	003 003	004 012	002 014	003 003	004 004	011 011	005 010
EP 1-04	002 002	003 003	011 013	022 022	011 012	005 014	003 012	001 004	006 012	004 010
EP 1-05	002 002	002 002	011 013	017 017	011 012	014 014	003 003	004 004	011 011	003 003
EP 1-06	002 002	002 002	006 006	003 003	004 011	010 014	009 019	003 004	001 011	002 002
EP 1-07	002 002	003 003	011 013	003 003	011 014	014 014	015 015	003 004	009 012	002 002
EP 1-08	002 002	003 003	016 017	013 016	004 011	002 002	003 003	001 001	011 011	009 009
EP 1-09	002 002	002 003	011 013	023 023	009 011	002 002	010 015	004 004	011 011	013 013
EP 1-10	002 002	002 003	008 008	022 022	004 011	014 014	015 015	001 003	001 001	003 004
EP 1-11	001 002	003 003	012 013	003 003	004 011	005 005	008 009	004 004	011 011	003 004
EP 1-12	002 002	003 003	011 011	004 004	009 009	006 006	009 009	004 004	011 011	004 005
EP 1-13	002 002	001 002	003 003	004 004	004 012	005 005	019 020	001 005	011 011	004 004
EP 1-14	002 002	003 003	003 003	003 003	009 011	014 014	010 019	004 004	011 011	009 009
EP 1-15	002 002	002 002	011 013	002 004	011 011	005 007	003 003	001 004	009 012	002 005
EP 1-16	002 002	002 002	013 019	003 003	014 014	007 007	004 004	004 004	009 012	003 004
EP 1-17	001 001	002 002	011 013	004 004	011 011	016 016	003 003	003 004	011 011	009 009
EP 1-18	002 002	002 002	011 019	029 029	005 011	005 005	015 015	004 004	011 012	005 009
EP 1-19	002 002	002 002	011 011	002 002	011 013	007 007	003 003	001 004	011 011	013 013
EP 1-20	001 002	001 001	011 011	010 010	011 011	002 002	003 003	001 004	009 009	004 009
EP 1-21	002 002	003 003	011 013	003 017	006 011	016 016	012 012	001 004	011 011	004 004

Appendix 4.1 continued

Ind	Locus1*	Locus2	Locus3*	Locus4*	Locus5	Locus6*	Locus7	Locus8*	Locus9*	Locus10
EP 1-22	001 002	002 002	013 016	009 019	011 011	016 016	003 003	001 003	006 006	002 003
EP 1-23	002 002	002 002	011 011	007 017	012 012	010 016	009 012	001 004	011 011	005 009
EP 1-24	002 002	003 003	011 011	010 010	005 011	002 014	008 008	003 004	011 011	003 006
EP 1-25	002 002	002 002	011 013	004 004	011 011	014 014	012 012	001 004	009 009	004 004
EP 1-26	002 002	002 002	013 014	003 022	011 011	014 014	003 004	001 001	011 011	004 004
EP 1-27	001 002	003 003	008 008	037 037	011 011	007 007	003 003	003 004	006 006	003 009
EP 1-28	002 002	002 002	016 019	019 019	011 011	002 014	016 016	001 001	011 011	004 004
EP 1-29	001 002	002 003	002 002	003 004	011 014	007 007	019 019	003 004	012 012	005 009
EP 1-30	001 002	002 003	003 003	004 004	011 011	002 005	025 025	003 003	012 012	004 004
EP 3-01	001 003	002 002	004 005	003 003	004 011	002 010	003 005	001 001	006 006	013 013
EP 3-02	001 002	002 002	011 011	003 007	011 011	007 007	015 018	004 004	009 009	002 004
EP 3-03	002 002	001 002	013 013	003 003	011 011	014 014	003 007	003 005	011 014	005 009
EP 3-04	001 001	002 002	003 003	010 024	011 011	009 009	003 003	003 003	004 011	005 011
EP 3-05	001 002	003 003	008 008	010 010	011 011	010 014	003 003	001 004	011 011	003 003
EP 3-06	002 002	001 002	004 008	003 010	011 011	002 002	003 003	001 001	011 012	005 005
EP 3-07	002 002	003 003	003 003	003 003	011 011	014 014	003 009	003 004	010 011	004 004
EP 3-08	002 002	001 002	013 013	003 003	011 011	014 014	003 007	003 005	011 014	005 009
EP 3-09	003 003	002 002	005 008	010 011	004 012	014 014	009 018	003 004	012 012	004 005
EP 3-10	002 002	003 003	004 004	007 007	011 011	005 007	003 010	001 005	011 011	013 013
EP 3-11	002 002	002 002	003 003	013 013	011 014	016 016	003 003	001 004	012 012	004 009
EP 3-12	002 002	002 002	008 008	003 003	011 011	006 006	004 004	001 004	011 011	002 002
EP 3-13	002 002	003 003	005 005	017 017	011 011	002 014	003 003	001 003	009 009	004 005
EP 3-14	002 002	003 003	005 005	003 007	004 011	005 007	003 007	004 005	009 011	011 013
EP 3-15	002 003	002 003	012 013	004 004	011 011	007 014	010 018	001 003	009 011	016 016
EP 3-16	002 002	002 003	008 011	003 007	007 011	015 015	020 020	001 004	012 012	004 011
EP 3-17	002 002	002 003	005 011	003 010	011 013	007 007	003 009	001 004	010 011	005 008
EP 3-18	002 002	001 002	011 011	003 003	004 011	006 010	003 003	003 004	012 012	004 004
EP 3-19	001 002	002 002	004 008	024 024	004 011	014 014	009 009	001 004	011 012	004 004
EP 3-20	002 002	002 002	002 002	003 003	009 011	014 014	003 003	004 004	006 006	004 005
EP 3-21	001 001	002 002	005 011	010 010	011 011	007 010	009 017	001 003	011 011	005 005
EP 3-22	002 002	002 003	004 004	010 010	011 012	009 014	019 021	003 004	011 014	005 013
EP 3-23	001 002	002 002	012 013	003 022	011 011	002 005	019 019	001 004	010 010	004 004
EP 3-24	001 002	002 003	003 003	003 007	011 011	010 010	003 003	001 003	004 008	002 002
EP 3-25	002 002	003 003	002 008	003 004	011 011	014 014	016 016	001 004	011 011	009 015
EP 3-26	002 002	002 002	011 011	002 010	011 013	010 014	003 003	004 004	012 012	006 006
EP 3-27	001 002	002 003	012 012	003 024	004 011	010 010	003 003	004 004	010 010	003 004
EP 3-28	001 002	002 002	008 008	017 017	002 002	014 014	001 008	003 004	012 012	003 003
EP 4-01	002 002	001 001	011 011	003 003	009 014	010 010	019 022	003 003	016 016	004 004
EP 4-02	002 002	003 003	011 014	004 004	011 012	005 007	003 003	001 004	011 011	004 004
EP 4-03	002 002	002 002	011 011	003 003	011 014	007 010	009 016	004 004	012 014	004 005
EP 4-04	002 002	002 002	006 006	022 022	004 009	002 006	009 009	003 003	008 012	004 004
EP 4-05	002 002	002 002	011 013	002 003	011 011	003 003	003 003	001 004	011 011	005 005
EP 4-06	002 002	003 003	006 006	003 003	004 011	011 014	003 003	001 003	011 016	004 005
EP 4-07	002 002	002 002	011 016	002 004	011 011	006 009	009 010	004 004	004 004	005 005
EP 4-08	002 002	001 001	013 016	004 019	010 011	007 007	010 010	001 004	009 011	004 005
EP 4-09	001 002	003 003	011 011	004 004	003 012	008 010	015 015	003 003	012 012	004 004
EP 4-10	002 002	003 003	013 017	003 003	004 012	007 010	009 009	004 004	016 016	004 004
EP 4-11	002 002	003 003	006 008	003 003	011 011	005 011	010 010	003 004	009 012	005 005
EP 4-12	002 002	001 001	006 011	004 010	011 011	005 005	009 010	001 003	016 016	004 004

Appendix 4.1 continued

Ind	Locus1*	Locus2	Locus3*	Locus4*	Locus5	Locus6*	Locus7	Locus8*	Locus9*	Locus10
EP 4-13	002 002	002 002	011 011	004 010	011 014	002 010	010 015	003 005	010 011	004 004
EP 4-14	002 002	003 003	011 013	004 004	002 002	005 007	015 015	004 004	010 011	004 004
EP 4-15	002 002	003 003	011 011	004 010	006 011	005 010	017 017	003 003	009 011	004 004
EP 4-16	002 002	002 002	011 016	002 010	004 011	002 002	023 028	002 004	009 009	003 007
EP 4-17	002 002	003 003	011 016	003 003	011 011	005 005	004 009	004 004	012 014	004 004
EP 4-18	002 002	003 003	011 016	003 003	011 011	002 010	003 007	003 004	012 012	005 005
EP 4-19	002 002	002 002	013 013	010 010	011 012	004 014	017 017	003 004	004 012	013 013
EP 4-20	002 002	002 002	003 003	022 026	009 011	014 014	010 010	003 004	004 009	013 013
EP 4-21	002 002	002 002	011 011	011 011	011 011	005 007	009 015	003 004	012 012	003 004
EP 4-22	002 002	001 002	011 020	041 041	011 011	002 011	003 003	003 004	011 014	004 005
EP 4-23	002 002	002 002	011 016	002 003	009 011	010 014	003 009	003 004	014 014	004 005
EP 4-24	001 003	001 003	006 011	012 019	003 011	009 014	006 006	004 004	008 008	003 003
EP 4-25	002 002	002 002	006 006	002 003	011 011	002 005	009 017	004 004	004 004	003 003
EP 4-26	002 002	001 001	003 003	010 010	011 014	005 011	017 017	004 004	012 012	003 004
EP 4-27	002 002	002 003	011 016	010 026	011 012	005 005	009 009	004 004	006 016	005 007
EP 4-28	002 002	003 003	003 003	003 003	012 012	007 014	009 015	004 004	004 004	003 006
EP 4-29	002 002	002 002	011 011	003 003	011 012	005 014	009 012	003 003	- -	002 005
EP 4-30	002 002	002 002	011 011	002 003	011 012	005 005	009 009	003 004	009 009	004 006
EP 5-01	002 002	002 002	011 011	003 004	011 011	005 005	005 005	004 005	011 011	005 005
EP 5-02	002 002	002 004	011 013	024 024	011 011	007 010	016 016	001 004	011 011	003 005
EP 5-03	002 002	003 003	003 003	004 004	011 013	010 010	009 010	004 004	011 011	002 004
EP 5-04	002 002	002 002	008 008	002 004	011 011	002 005	009 009	003 004	011 011	007 009
EP 5-05	002 002	001 002	008 008	004 007	011 011	005 014	010 016	001 004	011 011	001 001
EP 5-06	001 002	003 003	003 017	011 011	011 014	014 014	012 012	003 004	011 011	002 004
EP 5-07	001 002	001 003	016 016	010 010	004 012	007 010	003 003	004 004	011 011	005 010
EP 5-08	002 002	003 003	003 004	017 032	004 011	003 005	004 004	003 003	011 011	003 009
EP 5-09	002 002	002 004	008 008	003 017	004 012	005 005	015 019	001 004	011 012	003 005
EP 5-10	002 002	002 002	011 013	017 017	011 011	010 014	003 003	003 003	011 011	004 005
EP 5-11	002 002	003 003	008 011	010 010	004 011	014 014	010 011	004 004	011 012	005 005
EP 5-12	002 002	002 002	011 011	003 003	011 014	015 015	012 016	001 004	011 011	004 009
EP 5-13	001 002	003 003	002 002	030 030	011 011	005 005	009 016	003 004	011 011	004 004
EP 5-14	002 002	002 002	002 013	004 010	011 014	002 005	003 012	001 004	011 011	003 004
EP 5-15	002 002	003 003	003 008	003 003	011 013	002 005	003 003	003 004	009 009	001 001
EP 5-16	002 002	002 004	002 008	022 024	011 012	002 005	009 009	001 004	009 009	004 011
EP 5-17	002 002	001 002	008 013	004 004	004 011	005 005	005 009	001 003	011 011	009 009
EP 5-18	002 002	002 002	008 008	010 010	011 011	002 002	009 009	004 004	011 011	002 004
EP 5-19	002 002	003 003	002 013	010 037	011 014	014 014	003 009	003 004	011 011	002 004
EP 5-20	002 002	002 002	011 016	029 029	011 011	006 014	010 010	001 004	011 011	002 004
EP 5-21	002 002	003 003	008 017	002 002	011 011	005 014	003 003	004 004	011 011	004 007
EP 5-22	002 002	002 002	002 002	004 004	011 011	006 014	010 010	003 003	011 011	003 005
EP 5-23	001 002	004 004	011 016	016 017	011 011	006 006	009 019	001 004	009 011	004 005
EP 5-24	002 002	003 003	003 008	007 019	011 011	002 007	009 009	003 004	011 011	005 005
EP 5-25	002 002	003 003	011 011	004 011	011 012	005 014	010 019	003 004	011 011	005 005
EP 5-26	002 002	002 004	011 011	017 017	006 011	005 015	010 019	001 004	011 011	002 007
EP 5-27	002 002	002 002	008 008	002 004	010 011	005 016	008 009	003 004	011 011	007 009
EP 5-28	002 002	003 003	011 011	003 011	011 012	005 014	010 010	003 004	011 011	005 005
EP 5-29	001 002	003 003	003 017	011 011	011 014	014 014	012 019	003 004	011 011	002 004
EP 5-30	002 002	002 002	008 008	010 010	006 011	005 005	010 018	004 004	009 011	004 004

Appendix 4.1 continued

Ind	Locus1*	Locus2	Locus3*	Locus4*	Locus5	Locus6*	Locus7	Locus8*	Locus9*	Locus10
Mid-										
EP 1-31	001 001	002 002	011 013	003 003	011 012	016 016	003 003	001 001	011 011	004 005
EP 1-32	002 002	003 003	008 008	030 030	011 011	016 016	003 010	001 004	011 011	005 009
EP 1-33	002 002	002 002	011 011	003 003	004 011	010 014	019 019	004 005	011 011	002 002
EP 1-34	002 002	002 002	003 013	003 003	011 012	009 009	015 019	001 004	011 011	005 005
EP 1-35	002 002	002 002	008 008	001 002	011 011	005 005	012 012	003 004	009 011	003 004
EP 1-36	001 002	003 003	002 013	004 004	011 011	014 014	015 015	003 004	011 012	005 005
EP 1-37	002 002	001 001	008 008	003 003	012 012	006 006	003 003	001 004	011 011	004 004
EP 1-38	002 002	003 003	008 013	011 011	012 012	014 014	003 003	003 004	011 012	003 009
EP 1-39	002 002	002 002	005 011	003 010	011 011	003 005	003 003	004 004	011 011	004 004
EP 1-40	002 002	002 003	008 008	010 010	011 011	002 010	009 010	001 003	012 012	013 013
EP 1-41	002 002	003 003	003 008	004 004	012 012	007 007	019 019	001 004	011 011	004 004
EP 1-42	002 002	002 002	013 016	010 010	011 011	014 014	003 003	001 004	012 012	005 013
EP 1-43	001 002	002 002	003 013	024 024	009 011	002 005	003 007	001 004	011 011	003 008
EP 1-44	002 002	002 003	013 014	003 003	011 012	008 008	004 004	001 003	012 012	004 004
EP 1-45	002 003	002 002	004 016	003 010	011 011	005 007	009 010	001 003	011 011	002 002
EP 1-46	001 002	002 002	008 008	003 005	004 011	015 015	003 003	004 004	011 011	002 002
EP 1-47	002 002	004 004	011 011	004 004	011 011	007 007	003 003	004 004	011 011	006 009
EP 1-48	001 002	002 002	011 011	003 003	004 011	010 010	012 012	001 003	011 011	004 015
EP 1-49	002 002	002 002	013 013	003 011	006 011	006 014	008 009	001 003	011 011	003 005
EP 1-50	002 002	003 003	018 018	003 003	011 011	010 014	012 012	001 001	011 011	002 005
EP 1-51	002 002	001 003	011 011	017 017	009 009	002 010	009 009	003 003	009 010	003 003
EP 1-52	002 002	003 003	011 014	010 024	011 012	014 014	011 017	001 001	011 011	002 005
EP 1-53	002 002	002 002	011 013	010 011	011 013	005 014	005 005	004 004	011 012	001 003
EP 1-54	002 002	003 003	011 016	003 003	009 009	002 014	003 003	003 005	010 011	002 003
EP 1-55	002 003	003 003	008 011	003 003	004 011	015 015	003 003	004 004	011 012	002 010
EP 1-56	001 002	001 002	011 011	004 004	011 011	003 014	004 012	003 003	011 011	003 005
EP 1-57	002 002	003 003	008 008	003 011	004 011	007 007	019 019	004 004	009 012	005 005
EP 1-58	002 002	003 003	011 011	007 011	009 009	013 013	003 009	003 004	011 011	008 008
EP 1-59	002 002	001 004	011 014	019 019	009 009	003 005	012 019	003 003	011 011	003 005
EP 3-31	001 002	001 002	011 011	004 011	009 009	003 003	003 003	004 004	004 012	004 004
EP 3-32	002 002	002 003	013 013	032 032	011 015	007 007	003 003	004 004	011 012	003 003
EP 3-33	002 002	003 003	012 012	003 003	004 011	005 005	003 003	003 003	011 011	002 004
EP 3-34	002 002	002 002	008 008	003 011	011 011	007 010	005 005	001 003	011 011	004 005
EP 3-35	002 002	001 002	012 013	003 011	011 011	010 014	009 009	001 004	011 011	002 002
EP 3-36	001 002	002 002	008 013	017 017	011 011	005 007	004 004	001 004	011 012	002 005
EP 3-37	002 002	002 002	003 011	002 002	011 011	005 010	018 018	003 004	011 012	004 005
EP 3-38	002 002	002 002	002 008	034 034	011 011	006 010	003 003	001 004	012 012	004 006
EP 3-39	001 002	001 003	013 013	017 024	011 011	002 005	009 018	004 004	011 011	003 007
EP 3-40	002 003	003 003	008 012	017 030	011 011	012 014	020 020	001 004	012 012	003 004
EP 3-41	002 002	003 003	003 011	017 017	011 012	007 014	017 018	003 003	012 012	003 003
EP 3-42	002 002	003 003	003 003	022 024	011 011	006 010	003 003	003 003	011 011	005 009
EP 3-43	002 002	001 002	012 013	002 002	011 011	006 010	003 003	004 004	004 011	005 005
EP 3-44	002 002	002 002	011 012	003 003	011 011	016 016	003 003	003 003	010 011	003 004
EP 3-45	002 002	002 002	003 013	010 010	011 011	001 001	003 003	004 004	011 011	005 005
EP 3-46	001 002	003 003	016 019	026 037	011 011	005 005	014 018	001 004	011 011	004 005
EP 3-47	002 002	002 003	011 014	002 011	011 011	006 007	009 009	003 004	012 012	004 004
EP 3-48	002 002	001 002	006 011	034 034	011 011	007 007	015 019	003 003	007 007	005 009
EP 3-49	002 002	002 002	011 016	003 004	011 012	005 007	003 009	001 004	009 009	003 009

Appendix 4.1 continued

Ind	Locus1*	Locus2	Locus3*	Locus4*	Locus5	Locus6*	Locus7	Locus8*	Locus9*	Locus10
EP 3-50	001 002	002 003	016 016	003 011	011 011	003 009	003 016	004 004	012 014	003 008
EP 3-51	001 002	002 002	011 014	019 019	004 011	008 009	003 003	001 003	012 014	003 004
EP 3-52	001 001	002 002	004 008	004 017	004 012	014 014	003 003	001 004	011 011	005 005
EP 3-53	002 002	003 003	011 014	004 004	011 011	014 014	003 003	001 003	011 012	004 005
EP 3-54	001 001	002 002	011 013	003 024	011 011	010 010	007 009	001 005	011 011	004 004
EP 3-55	002 002	002 003	011 011	013 013	011 011	003 005	010 010	003 004	012 012	004 005
EP 3-56	002 003	001 001	011 011	010 017	007 011	002 007	017 021	001 003	011 011	005 009
EP 3-57	001 002	002 003	013 017	016 017	005 005	002 007	018 018	001 004	011 011	003 004
EP 3-58	002 002	002 002	004 016	003 011	011 011	002 009	003 004	004 004	010 011	004 004
EP 3-59	002 002	003 003	003 006	003 004	005 011	011 014	003 003	003 004	011 011	009 009
EP 3-60	002 002	002 002	008 011	013 013	011 012	016 016	010 015	003 004	011 011	004 005
EP 4-31	002 002	003 003	011 011	010 010	009 011	002 008	003 003	005 005	014 014	002 002
EP 4-32	002 002	002 003	003 003	003 003	009 014	007 007	010 013	001 001	009 009	002 004
EP 4-33	001 002	002 003	011 013	002 003	011 014	002 011	010 010	003 004	012 012	005 009
EP 4-34	002 002	003 003	003 003	004 004	011 011	006 007	003 003	003 004	009 009	004 005
EP 4-35	002 002	002 003	011 011	010 011	011 011	011 014	003 003	001 004	011 011	014 014
EP 4-36	002 002	003 003	002 007	003 003	011 012	009 009	003 010	003 003	012 012	004 004
EP 4-37	002 002	003 003	011 011	010 039	004 011	002 010	010 011	004 005	011 011	005 005
EP 4-38	002 002	003 003	011 013	002 010	009 014	016 016	004 009	004 004	004 004	004 004
EP 4-39	002 002	002 002	011 011	004 004	004 011	007 007	008 010	003 004	012 012	015 015
EP 4-40	002 002	002 002	013 013	002 012	009 009	016 016	017 024	004 004	004 004	002 005
EP 4-41	002 002	003 003	003 010	037 037	003 011	014 014	009 009	001 003	010 012	002 002
EP 4-42	002 002	002 002	014 014	002 010	011 011	016 016	003 003	003 003	004 012	005 005
EP 4-43	002 002	003 003	011 011	004 004	005 011	005 014	003 003	003 003	004 010	003 005
EP 4-44	002 002	002 005	006 013	004 010	004 011	005 010	018 018	001 005	012 014	002 002
EP 4-45	002 002	003 003	014 016	003 004	011 011	005 005	010 016	003 003	006 012	004 005
EP 4-46	002 002	002 002	003 003	010 022	011 011	003 006	018 018	003 004	009 012	004 004
EP 4-47	002 002	003 003	003 003	004 004	011 011	003 003	009 009	004 004	004 004	004 005
EP 4-48	002 002	001 003	005 006	004 005	011 011	005 005	009 010	003 004	009 012	005 005
EP 4-49	002 002	002 003	006 006	012 012	011 011	007 007	017 017	003 004	009 012	004 004
EP 4-50	002 002	003 003	013 016	003 003	010 011	005 007	013 013	004 004	009 009	004 004
EP 4-51	002 002	002 002	006 016	010 011	011 011	007 010	009 009	003 003	004 009	002 003
EP 4-52	002 002	002 002	008 008	003 003	004 011	014 014	003 003	004 004	009 009	003 005
EP 4-53	002 002	003 003	006 006	006 006	009 014	016 016	009 009	003 004	009 016	004 004
EP 4-54	002 002	002 002	003 004	004 004	011 011	005 010	016 018	004 004	011 011	001 001
EP 4-55	002 002	001 001	011 011	003 003	011 011	010 010	004 004	004 004	004 010	002 005
EP 4-56	001 002	002 002	013 016	003 005	011 014	002 002	015 015	003 003	012 014	004 004
EP 4-57	002 002	003 003	004 014	002 002	009 014	002 006	009 009	004 004	006 012	002 002
EP 4-58	001 002	003 003	005 005	003 004	010 011	011 011	003 003	003 005	009 012	004 005
EP 4-59	002 002	002 002	008 008	003 003	004 011	014 014	003 003	004 004	009 009	003 005
EP 4-60	002 002	001 001	011 011	003 022	009 011	009 009	003 003	003 004	011 012	004 011
EP 5-31	001 002	001 002	013 016	004 010	006 011	002 014	009 009	004 004	012 012	009 009
EP 5-32	002 002	003 003	011 013	017 017	004 011	005 005	015 015	001 004	011 011	004 005
EP 5-33	002 002	001 002	005 005	004 004	016 016	009 009	016 019	003 004	011 011	007 007
EP 5-34	001 001	002 004	013 016	004 017	011 011	009 009	019 019	004 004	011 011	004 009
EP 5-35	002 002	003 003	011 011	010 010	011 011	015 016	018 019	003 004	011 011	004 005
EP 5-36	002 002	002 003	008 008	003 004	011 011	016 016	009 009	004 004	011 011	002 002
EP 5-37	002 002	002 002	011 016	007 007	011 011	002 002	009 014	001 004	011 011	004 005

Appendix 4.1 continued

Ind	Locus1*	Locus2	Locus3*	Locus4*	Locus5	Locus6*	Locus7	Locus8*	Locus9*	Locus10
EP 5-38	002 002	001 002	016 016	003 022	011 011	002 002	012 018	003 004	009 011	012 015
EP 5-39	002 002	002 002	005 005	017 017	004 011	009 009	019 019	001 004	011 011	005 009
EP 5-40	002 002	002 002	011 016	004 004	006 011	009 014	016 019	004 004	012 012	004 004
EP 5-41	002 002	002 003	006 009	003 003	011 011	003 005	010 010	004 004	011 011	009 009
EP 5-42	002 002	002 002	008 008	002 002	011 011	007 014	003 003	001 003	011 012	002 004
EP 5-43	001 002	003 003	014 016	003 004	011 013	016 016	010 012	003 004	011 012	004 005
EP 5-44	002 002	002 003	011 011	003 003	004 011	005 014	010 016	004 004	011 011	004 004
EP 5-45	002 002	002 002	016 016	003 003	012 012	005 014	009 009	001 003	012 012	002 003
EP 5-46	002 002	002 002	002 011	004 010	011 013	015 015	003 003	004 004	011 011	002 002
EP 5-47	002 002	002 002	011 013	003 011	004 011	007 007	003 003	004 004	011 011	002 003
EP 5-48	002 002	002 004	008 008	003 007	004 011	003 005	003 003	003 004	011 012	001 001
EP 5-49	002 002	001 003	013 013	003 003	011 011	016 016	003 005	001 003	009 009	014 014
EP 5-50	002 002	001 001	002 002	004 010	011 011	015 015	002 012	004 004	011 011	002 002
EP 5-51	001 002	002 002	008 011	004 004	011 014	002 002	003 003	004 004	011 011	004 005
EP 5-52	001 002	002 002	004 014	003 003	009 011	002 009	010 010	003 004	011 011	004 005
EP 5-53	002 002	003 003	013 016	002 003	011 011	009 009	004 009	001 004	011 011	003 006
EP 5-54	002 002	002 003	011 013	004 017	004 012	006 014	003 003	001 003	012 012	004 005
EP 5-55	002 002	002 002	002 011	024 024	011 013	005 014	005 009	004 004	009 011	011 011
EP 5-56	002 002	002 002	003 003	011 017	011 011	002 002	003 005	004 004	011 011	003 003
EP 5-57	001 002	003 003	016 016	003 003	004 011	002 007	003 010	001 004	011 012	002 004
EP 5-58	002 002	002 002	013 013	004 004	011 014	005 005	016 018	004 004	011 012	003 004
EP 5-59	002 002	001 002	008 008	003 003	012 012	003 003	004 004	001 004	011 011	002 002
EP 5-61	002 002	003 003	013 016	017 017	011 011	002 007	019 019	003 004	011 011	002 002
EP 5-62	002 002	002 002	011 013	003 017	004 011	016 016	016 019	001 004	011 011	002 002
EP 5-63	002 002	002 005	011 016	004 024	011 012	005 005	009 009	001 004	012 012	003 005
EP 5-64	002 002	003 003	011 011	003 010	- -	010 010	003 003	004 004	011 011	004 004
EP 5-65	002 002	002 002	011 014	004 004	004 011	005 005	003 003	001 004	011 011	004 004
EP 5-66	002 002	003 003	001 001	005 017	011 012	010 010	010 015	003 004	001 011	009 009
EP 5-67	002 002	002 003	008 008	017 017	011 013	010 014	010 011	004 004	012 012	007 007
EP 5-68	002 002	002 002	006 011	003 005	009 009	014 014	009 009	004 004	011 011	004 004
EP 5-69	002 002	002 002	013 014	032 032	004 011	002 005	009 015	003 004	011 012	005 005
EP 5-70	002 002	002 002	003 016	017 017	011 014	006 006	008 026	001 004	011 011	005 005
EP 5-71	002 002	002 003	011 013	003 003	011 011	005 014	015 016	004 004	011 012	001 001
EP 5-72	001 001	002 002	008 013	003 003	011 011	014 014	003 003	004 004	011 011	009 009
EP 5-73	002 002	002 004	005 011	003 003	011 011	005 005	005 005	004 004	010 010	004 004
EP 5-74	002 002	003 003	008 011	004 004	011 011	006 014	019 019	001 001	012 012	004 004
EP 5-75	002 002	002 002	002 003	011 017	011 011	007 007	003 018	001 004	011 011	009 009
EP 5-76	002 002	001 002	011 013	037 037	004 011	005 006	010 010	004 004	011 011	004 005
EP 5-77	002 002	003 003	003 003	017 017	011 011	005 005	003 003	004 004	009 011	003 011
EP 5-78	002 002	002 002	011 016	004 007	011 012	003 006	003 003	004 004	011 011	004 004
EP 5-79	002 002	002 002	011 011	011 011	011 011	005 014	005 005	003 004	011 011	005 005
EP 5-80	001 002	003 003	006 006	003 003	004 011	005 014	010 010	001 004	011 012	008 008
EP 5-81	002 002	002 002	006 011	003 003	004 011	016 016	010 010	004 004	011 011	004 011
EP 5-82	002 002	002 002	008 008	002 002	011 011	007 014	003 003	001 003	011 012	002 004
EP 5-83	002 002	003 003	005 005	017 017	011 011	009 009	012 012	001 001	011 011	004 004
EP 5-84	001 002	003 003	016 016	004 010	004 011	005 005	004 009	004 004	011 011	004 004
EP 5-85	001 002	003 003	002 008	005 022	016 016	005 014	003 003	001 004	009 009	003 004
EP 5-86	002 002	003 003	016 016	010 010	011 011	002 002	005 005	004 004	011 011	005 005
EP 5-87	002 002	003 003	005 005	004 017	009 011	005 014	016 016	003 004	010 011	004 004

Appendix 4.1 continued

Ind	Locus1*	Locus2	Locus3*	Locus4*	Locus5	Locus6*	Locus7	Locus8*	Locus9*	Locus10
EP 5-88	001 002	002 002	011 013	005 005	004 011	003 003	016 016	003 003	011 011	005 005
EP 5-89	002 002	003 003	011 013	017 022	006 011	005 010	005 009	003 004	011 011	003 003
Late										
EP 1-61	002 002	002 002	011 018	010 010	004 011	004 007	003 009	001 001	011 011	003 005
EP 1-62	002 002	003 003	017 017	002 010	004 011	005 005	003 009	004 004	011 011	003 005
EP 1-63	001 002	002 002	011 018	004 010	004 011	004 007	009 009	001 001	012 012	004 004
EP 1-64	002 002	002 003	011 011	004 004	005 011	002 002	003 015	001 003	011 011	005 009
EP 1-65	002 002	003 003	002 002	003 003	004 011	002 002	004 019	001 003	011 012	004 005
EP 1-66	002 002	003 003	011 011	003 003	011 011	016 016	003 016	003 004	011 011	005 006
EP 1-67	002 002	003 003	004 011	003 003	003 011	009 010	005 015	001 001	009 009	004 005
EP 1-68	002 003	003 003	004 011	004 004	003 011	009 010	027 027	001 001	011 014	002 011
EP 1-69	002 002	003 003	016 016	002 010	011 011	002 002	003 015	001 001	011 011	005 009
EP 1-70	002 002	003 003	017 017	002 010	004 011	005 005	005 015	004 004	009 009	004 005
EP 1-71	002 002	003 003	008 013	032 037	011 011	002 005	004 008	001 003	011 011	003 004
EP 1-72	002 002	002 002	011 014	017 029	011 011	016 016	004 008	001 004	011 011	004 004
EP 1-73	001 002	003 003	004 011	002 022	011 011	002 002	003 013	003 003	011 012	003 005
EP 1-74	001 002	002 002	008 013	004 004	004 011	002 006	003 009	001 004	009 011	005 007
EP 1-75	001 002	002 002	008 013	003 003	011 011	014 014	003 009	001 004	011 012	003 005
EP 1-76	002 002	003 003	002 011	003 010	011 013	007 014	003 009	001 004	010 010	003 004
EP 1-77	002 002	002 002	008 011	002 002	011 011	014 014	009 009	003 004	009 009	003 013
EP 1-78	002 003	002 002	016 019	002 010	011 012	016 016	005 005	001 004	011 011	004 004
EP 1-79	002 002	003 003	011 011	004 017	004 012	009 009	008 008	004 004	011 011	004 013
EP 1-80	002 002	003 003	011 013	004 004	011 011	002 014	004 016	003 004	011 011	004 006
EP 1-81	002 002	003 003	011 011	004 004	011 011	002 014	008 018	003 004	001 001	013 013
EP 1-82	002 002	003 003	012 016	011 011	011 011	002 002	008 018	001 005	011 011	003 013
EP 1-83	002 002	003 003	011 016	017 017	004 011	016 016	004 009	001 004	011 011	002 005
EP 1-84	002 002	002 002	011 011	002 002	004 011	005 014	003 009	003 004	011 011	005 009
EP 1-85	002 002	003 003	011 013	002 002	011 011	005 007	009 009	001 004	011 011	003 013
EP 1-86	002 002	002 002	003 013	003 003	006 011	007 014	003 015	001 004	012 012	003 003
EP 1-87	001 002	001 002	004 012	010 010	011 014	002 007	013 013	004 004	011 011	003 006
EP 1-88	001 002	004 004	011 011	010 010	011 013	005 010	003 019	004 004	011 011	004 005
EP 3-61	001 002	003 003	011 016	005 012	011 011	005 014	003 021	001 004	011 011	003 006
EP 3-62	002 002	002 003	011 011	010 010	011 011	009 014	003 012	001 004	012 012	003 003
EP 3-63	001 002	003 003	008 011	003 003	009 009	009 009	003 010	004 004	009 011	004 004
EP 3-64	002 002	003 003	011 013	005 018	011 011	014 014	018 018	004 004	011 014	004 004
EP 3-65	001 003	003 003	005 013	008 012	011 011	014 014	003 009	001 004	011 011	004 005
EP 3-66	002 002	002 002	008 013	005 005	011 012	009 016	003 003	001 004	004 011	003 003
EP 3-67	001 002	002 002	003 013	004 005	011 011	005 014	009 019	003 004	004 011	004 010
EP 3-68	001 002	002 002	011 011	004 014	011 011	002 002	019 020	001 004	011 011	005 005
EP 3-69	002 002	002 002	011 012	004 012	- -	014 014	004 019	004 004	004 004	009 009
EP 3-70	002 002	003 003	011 012	003 005	011 012	010 014	009 011	003 004	011 012	004 005
EP 3-71	002 003	003 003	003 012	032 037	011 011	002 002	009 015	001 003	011 012	003 003
EP 3-72	002 002	002 003	011 014	008 011	011 011	002 003	003 012	003 004	011 014	003 005
EP 3-73	001 002	002 003	011 011	004 011	011 011	007 014	003 021	003 004	011 011	003 006
EP 3-74	002 002	003 003	011 016	005 012	011 011	005 014	009 018	001 004	001 011	004 004
EP 3-75	001 003	003 003	009 011	003 003	011 014	005 007	009 015	001 001	012 012	002 003
EP 3-76	001 002	003 003	004 008	008 008	011 011	006 006	009 015	004 004	012 012	005 009
EP 3-77	002 002	003 003	011 012	003 003	012 012	007 007	003 020	003 003	009 009	003 004
EP 3-78	002 002	002 003	004 008	004 004	010 011	002 014	003 017	001 004	011 011	005 005

Appendix 4.1 continued

Ind	Locus1*	Locus2	Locus3*	Locus4*	Locus5	Locus6*	Locus7	Locus8*	Locus9*	Locus10
EP 3-79	002 002	003 003	011 011	004 004	011 011	010 010	017 018	001 001	009 012	004 004
EP 3-80	002 002	002 002	008 013	012 023	011 011	002 010	003 010	004 004	012 012	005 005
EP 3-81	002 002	002 002	003 003	004 004	011 012	014 014	009 017	001 004	011 011	004 005
EP 3-82	002 002	002 002	011 012	012 012	011 013	005 005	003 016	001 004	011 011	005 009
EP 3-83	002 002	002 002	011 012	012 012	011 011	005 005	003 015	004 004	004 011	005 009
EP 3-84	003 003	002 003	016 016	004 010	011 011	010 016	003 009	001 003	011 011	004 004
EP 3-85	003 003	002 003	016 016	004 010	011 011	010 016	003 009	001 003	011 011	004 004
EP 3-86	001 001	003 003	002 005	004 029	- -	005 010	003 016	004 005	001 001	002 003
EP 3-87	002 003	002 002	011 013	020 029	011 012	015 016	015 019	004 004	001 001	003 009
EP 3-88	002 002	003 003	004 013	007 017	009 012	002 005	008 018	001 003	012 012	004 013
EP 4-61	002 002	003 003	011 013	004 011	012 012	005 014	010 015	003 003	009 009	003 004
EP 4-62	002 002	002 003	011 011	004 004	011 011	- -	009 009	003 003	009 012	002 005
EP 4-63	002 002	003 003	012 013	004 004	011 011	002 002	010 010	001 004	011 011	004 013
EP 4-64	002 002	003 003	004 011	004 004	011 011	002 002	010 017	001 004	010 010	004 009
EP 4-65	001 002	003 003	011 011	004 004	009 011	002 002	010 015	001 004	012 012	004 005
EP 4-66	002 002	002 002	003 017	003 003	003 012	010 014	005 016	001 003	012 012	003 004
EP 4-67	002 002	003 003	011 011	005 011	004 011	005 014	011 024	001 004	009 009	004 005
EP 4-68	002 002	001 001	011 011	025 025	011 012	006 010	009 016	003 004	009 012	002 006
EP 4-69	002 002	003 003	004 006	012 031	003 011	002 005	004 024	003 003	012 012	005 013
EP 4-70	002 002	003 003	004 006	004 005	011 014	005 005	009 012	003 004	011 012	002 005
EP 4-71	002 002	002 003	003 011	004 011	011 014	009 016	010 017	003 004	011 011	004 004
EP 4-72	002 002	003 003	003 003	010 010	003 011	005 005	010 011	001 005	001 001	005 005
EP 4-73	002 002	002 002	011 011	005 026	011 012	010 014	009 009	003 003	009 009	002 005
EP 4-74	001 002	002 002	006 016	003 011	011 014	006 014	018 019	001 004	012 012	004 004
EP 4-75	002 002	002 003	004 011	003 003	011 011	- -	010 017	001 004	010 012	004 009
EP 4-76	002 002	003 003	011 011	041 041	009 011	002 002	009 019	003 005	003 006	003 003
EP 4-77	002 002	001 002	003 003	004 004	011 013	014 014	007 017	003 004	009 011	005 013
EP 4-78	002 002	001 001	003 011	004 004	004 011	005 005	016 028	003 004	009 009	006 006
EP 4-79	002 002	002 002	011 016	004 023	011 011	002 002	003 017	003 003	004 009	004 004
EP 4-80	002 002	002 002	011 011	005 011	011 014	007 010	009 019	003 004	011 011	003 005
EP 4-81	002 002	003 003	011 013	017 030	004 011	005 005	003 003	001 004	011 012	005 005
EP 4-82	002 002	001 002	003 005	004 005	011 011	005 005	009 010	004 004	011 012	004 004
EP 4-83	002 002	003 003	011 014	030 037	009 015	005 014	010 026	001 004	011 011	004 005
EP 4-84	002 002	002 003	003 013	004 011	011 014	002 009	012 015	003 004	012 012	005 006
EP 4-85	002 002	003 003	004 006	012 031	003 011	002 005	010 017	003 003	011 012	004 004
EP 4-86	002 002	001 001	011 011	004 005	011 011	005 010	009 016	003 003	009 009	002 002
EP 4-87	002 002	002 002	004 004	026 026	004 011	002 014	015 019	001 001	012 012	003 005
EP 4-88	002 002	002 002	003 011	010 022	003 011	002 006	009 014	001 003	009 012	004 004
EP 4-89	001 002	003 003	003 011	003 004	011 011	007 007	003 009	004 004	011 014	003 004
EP 4-90	002 002	003 003	005 006	004 004	011 014	005 009	009 024	003 005	012 012	005 005
EP 5-91	002 002	003 003	003 013	017 017	004 011	005 005	003 003	004 004	009 011	003 011
EP 5-92	002 002	002 003	003 011	003 005	- -	005 010	009 009	001 004	012 012	003 003
EP 5-93	002 002	002 004	008 016	008 008	004 011	005 005	003 019	004 004	011 012	013 013
EP 5-94	002 002	002 002	003 006	005 005	006 011	005 014	003 016	001 003	011 011	002 002
EP 5-95	001 002	002 002	002 013	005 031	011 011	014 016	009 009	001 003	011 012	003 011
EP 5-96	003 003	002 003	011 013	004 012	011 011	014 014	003 003	004 004	010 012	003 004
EP 5-97	002 002	002 002	002 011	004 004	013 013	005 005	011 019	003 004	010 010	001 013
EP 5-98	002 002	002 002	002 011	005 005	011 013	005 016	011 019	003 004	010 010	003 003

Appendix 4.1 continued

Ind	Locus1*	Locus2	Locus3*	Locus4*	Locus5	Locus6*	Locus7	Locus8*	Locus9*	Locus10
EP 5-99	001 002	003 003	016 016	003 003	011 011	007 007	003 016	004 004	011 011	009 013
EP 5-100	002 002	003 003	006 011	004 005	011 014	003 010	003 003	003 003	- -	003 003
EP 5-101	001 002	002 002	011 012	005 006	004 011	014 014	003 003	001 003	011 011	004 004
EP 5-102	002 002	002 004	008 011	004 012	011 014	014 014	014 016	004 004	011 011	008 009
EP 5-103	002 002	003 003	003 016	032 032	004 011	006 016	008 018	003 004	011 011	004 004
EP 5-104	002 002	002 004	011 019	018 018	006 011	005 005	010 019	001 004	011 011	002 007
EP 5-105	002 002	002 002	002 011	006 006	004 011	006 007	009 019	003 004	011 012	003 004
EP 5-106	002 002	003 003	011 011	004 004	011 011	015 016	019 019	004 004	011 012	006 006
EP 5-107	002 002	002 002	014 014	004 005	011 013	005 014	003 010	004 004	009 009	005 005
EP 5-108	002 002	002 002	011 013	030 030	006 011	005 014	009 009	004 004	011 011	004 009
EP 5-109	001 001	001 002	011 016	005 011	011 011	005 005	003 003	004 004	011 011	009 009
EP 5-110	002 002	003 003	013 013	004 004	011 011	016 016	003 009	001 004	011 011	002 004
EP 5-111	002 002	003 003	013 017	003 003	011 014	014 016	003 019	001 003	011 011	001 004
EP 5-112	002 002	002 002	011 016	011 011	011 011	003 003	003 019	003 004	011 011	003 009
EP 5-113	002 002	002 002	011 011	018 018	004 011	002 005	010 015	001 003	011 012	005 005
EP 5-114	002 002	003 003	011 011	004 004	011 011	015 016	019 019	004 004	011 012	006 006
EP 5-115	002 002	002 002	003 003	004 004	004 011	005 005	009 014	004 004	011 011	003 003
EP 5-116	002 002	002 004	013 016	010 010	011 014	005 005	005 009	003 004	009 011	004 005
EP 5-117	002 002	002 002	011 016	011 017	004 011	007 007	004 009	004 004	011 011	003 009
EP 5-118	001 002	002 003	003 011	010 022	004 011	006 006	008 015	001 001	009 010	009 009
2011										
EP 1-01	002 002	002 002	013 016	004 004	004 011	009 010	003 003	004 004	010 011	009 013
EP 1-02	002 002	002 003	011 012	004 389	011 014	002 005	009 019	001 004	011 011	011 011
EP 1-03	001 002	002 002	003 008	003 011	011 011	010 010	009 009	003 004	011 011	005 009
EP 1-04	002 002	002 003	013 018	004 011	011 011	007 010	003 003	001 004	011 011	003 005
EP 1-05	002 002	001 001	013 013	029 030	011 011	005 014	003 003	001 004	009 011	004 005
EP 1-06	002 002	001 001	008 008	003 003	012 012	006 006	003 003	001 004	011 011	004 004
EP 1-07	002 002	002 002	008 012	004 004	004 012	005 010	009 019	003 004	009 011	005 005
EP 1-08	001 001	001 001	011 011	010 024	011 011	014 014	003 003	001 004	011 012	004 009
EP 1-09	001 001	002 002	002 011	003 037	004 011	014 014	010 017	003 004	011 012	009 013
EP 1-10	002 002	002 003	002 003	003 004	004 011	005 014	009 019	004 004	009 012	002 002
EP 1-11	001 002	002 002	011 016	003 003	011 011	015 017	010 010	001 001	011 011	004 004
EP 1-12	002 002	002 002	011 011	005 010	003 008	005 014	003 010	003 004	011 011	002 002
EP 1-13	001 002	001 003	004 011	024 037	011 011	006 009	005 005	001 003	011 011	004 004
EP 1-14	001 002	001 004	003 011	003 032	004 012	016 016	009 009	001 001	009 012	004 013
EP 1-15	002 002	002 003	016 016	004 004	011 012	007 014	009 009	001 004	011 012	004 009
EP 1-16	001 002	002 002	011 013	004 004	004 012	002 014	009 016	001 004	011 011	009 009
EP 1-17	002 002	002 002	004 013	005 010	011 012	002 010	010 018	001 003	011 012	004 009
EP 1-18	002 002	002 002	002 011	004 011	011 011	002 005	009 010	003 004	011 011	005 005
EP 1-19	002 002	002 003	011 013	004 004	011 011	002 014	003 015	003 004	012 012	004 004
EP 1-20	001 002	002 002	004 016	003 010	011 013	005 007	009 010	001 003	011 011	002 002
EP 1-21	002 003	002 002	002 008	003 010	009 009	017 017	008 017	001 004	010 010	002 004
EP 1-22	001 002	002 002	013 013	007 007	009 011	002 009	009 019	001 003	011 011	003 009
EP 1-23	002 002	002 002	011 011	004 004	011 012	005 010	009 015	003 004	011 012	005 005
EP 3-01	002 002	002 003	003 013	003 013	011 011	014 014	003 009	003 004	010 011	004 004
EP 3-02	001 001	002 002	008 013	004 029	004 011	002 014	003 003	004 004	011 011	002 006
EP 3-03	001 002	003 004	005 005	004 024	004 011	014 014	003 010	004 004	010 010	005 005
EP 3-04	002 003	002 004	003 016	003 031	011 011	003 009	010 022	001 003	011 011	003 003
EP 3-05	001 002	003 003	003 011	037 037	005 011	014 014	010 015	003 003	011 012	002 004

Appendix 4.1 continued

Ind	Locus1*	Locus2	Locus3*	Locus4*	Locus5	Locus6*	Locus7	Locus8*	Locus9*	Locus10
EP 3-06	002 002	002 002	002 012	007 010	009 011	005 005	009 019	003 004	012 012	004 006
EP 3-07	001 002	002 003	011 013	002 004	005 011	002 014	003 015	003 004	011 011	004 218
EP 3-08	001 002	002 002	008 011	002 004	011 011	010 010	005 019	001 001	009 009	003 004
EP 3-09	002 002	003 003	005 005	017 017	011 011	002 014	003 003	001 003	009 009	004 005
EP 3-10	002 002	001 002	005 016	003 029	006 013	016 017	003 003	001 003	004 004	005 006
EP 3-11	002 002	002 002	005 013	003 032	011 012	016 016	005 009	001 003	012 012	003 004
EP 3-12	002 002	001 002	003 013	013 013	011 014	016 016	003 003	001 004	012 012	004 009
EP 3-13	002 002	001 003	011 011	003 012	011 014	002 007	003 014	004 004	011 011	002 004
EP 3-14	002 002	002 002	008 014	003 018	011 012	009 010	012 019	001 004	012 012	004 004
EP 3-15	002 002	002 002	011 011	003 013	011 013	003 010	013 018	004 004	011 011	005 009
EP 3-16	001 002	003 003	005 005	017 017	011 011	002 014	003 009	001 003	009 009	004 005
EP 3-17	001 002	003 003	004 013	005 010	011 011	014 014	015 015	003 004	011 011	004 004
EP 3-18	002 002	002 002	011 011	003 011	010 011	007 014	015 019	004 004	011 012	004 005
EP 3-19	002 002	002 003	003 013	003 003	011 011	014 014	003 009	003 004	010 011	004 004
EP 3-20	002 002	001 002	003 013	004 017	011 014	016 016	003 016	001 004	012 012	004 009
EP 3-21	002 002	001 002	011 011	003 010	009 009	010 010	019 019	003 004	012 012	002 004
EP 3-22	002 002	001 002	004 008	003 010	011 011	002 002	003 003	001 001	011 012	005 005
EP 3-23	002 002	002 002	002 011	002 005	011 014	005 014	015 015	003 004	011 014	004 004
EP 3-24	002 002	001 002	011 011	003 011	011 011	007 010	003 003	004 004	011 011	003 005
EP 3-25	002 002	002 002	011 011	020 020	012 012	005 009	009 010	003 004	012 012	005 005
EP 4-01	002 002	003 003	006 011	037 041	011 014	002 005	009 009	001 004	009 009	002 002
EP 4-02	002 002	002 003	008 011	002 003	011 014	002 015	007 009	003 004	011 016	005 218
EP 4-03	002 002	003 003	014 017	002 295	009 014	002 006	015 018	004 004	012 012	004 005
EP 4-04	002 003	001 002	011 011	002 003	011 011	002 009	009 015	004 004	012 012	002 002
EP 4-05	002 002	001 002	002 013	003 037	004 004	005 005	003 014	003 003	009 009	002 002
EP 4-06	001 002	002 003	006 011	003 010	003 011	003 011	009 017	003 004	012 012	004 004
EP 4-07	001 002	001 002	011 016	003 030	011 011	007 010	006 010	003 004	012 012	002 004
EP 4-08	002 002	001 001	011 011	003 004	011 014	006 006	009 027	003 003	012 012	004 004
EP 4-09	001 002	002 003	005 011	002 009	011 014	010 010	009 009	004 004	009 011	005 005
EP 4-10	002 002	002 002	011 011	003 041	011 011	005 014	009 015	001 004	012 012	004 004
EP 4-11	002 002	002 002	011 011	004 012	011 014	010 010	009 016	003 004	011 012	004 004
EP 4-12	002 002	001 002	005 013	002 004	008 014	005 011	016 017	003 004	011 012	004 004
EP 4-13	002 002	002 003	003 013	004 004	011 014	002 005	008 018	001 004	011 012	002 007
EP 4-14	001 002	001 001	003 019	002 004	009 011	002 002	018 024	004 004	012 012	004 013
EP 4-15	001 002	002 002	003 005	004 033	009 011	005 005	009 014	004 004	009 009	004 005
EP 4-16	002 002	002 002	011 013	010 010	003 009	007 007	010 016	003 004	012 012	002 002
EP 4-17	001 002	001 003	009 012	010 037	011 011	005 014	005 009	003 003	011 012	004 004
EP 4-18	002 002	002 002	004 011	003 022	011 014	014 014	003 010	003 004	009 011	003 003
EP 4-19	002 002	002 002	011 016	- -	011 011	002 003	015 015	003 004	012 012	005 005
EP 4-20	001 002	003 003	005 014	003 003	004 011	002 007	003 015	003 004	011 011	004 004
EP 4-21	002 002	001 001	011 012	011 025	011 011	002 014	003 003	003 003	009 012	002 004
EP 4-22	002 002	001 001	011 011	019 019	011 014	005 005	009 015	004 004	012 012	002 002
EP 4-23	002 002	003 003	003 013	003 004	011 014	007 007	005 005	004 004	012 012	004 004
EP 4-24	002 002	001 001	003 003	003 003	003 005	006 010	010 015	001 004	012 012	002 002
EP 4-25	002 002	002 002	005 006	002 035	011 011	002 002	003 003	001 001	004 004	004 005
EP 4-26	002 002	002 002	004 011	003 022	011 014	014 014	003 010	003 004	009 011	003 003
EP 5-01	001 002	002 003	011 011	024 024	004 011	003 010	010 015	003 004	009 009	005 009
EP 5-02	002 002	002 004	003 011	004 021	011 013	002 002	003 004	003 004	011 011	001 001

Appendix 4.1 continued

Ind	Locus1*	Locus2	Locus3*	Locus4*	Locus5	Locus6*	Locus7	Locus8*	Locus9*	Locus10
EP 5-03	002 002	002 003	011 013	004 010	011 011	005 005	003 019	001 004	011 011	009 009
EP 5-04	001 001	002 002	003 011	010 017	011 012	005 007	003 019	003 004	011 011	002 005
EP 5-05	002 002	003 003	011 013	010 026	011 011	009 009	014 014	004 004	011 012	002 004
EP 5-06	001 002	003 003	008 014	003 011	011 013	009 009	009 009	001 001	011 011	003 004
EP 5-07	002 002	002 002	002 016	003 011	004 011	016 016	016 016	004 004	009 011	004 004
EP 5-08	002 002	002 003	003 011	003 017	010 011	005 010	009 009	004 004	011 014	002 002
EP 5-09	002 002	002 003	011 013	003 003	011 011	005 014	015 016	004 004	011 012	001 004
EP 5-10	001 002	002 002	008 011	017 024	011 014	016 016	016 018	004 004	011 011	004 004
EP 5-11	002 002	002 002	002 003	002 017	011 012	010 010	003 008	003 004	009 011	003 010
EP 5-12	002 002	002 002	011 016	026 026	012 014	010 010	016 016	001 003	011 011	004 005
EP 5-13	002 002	002 002	003 008	017 017	011 014	002 002	009 019	001 003	012 012	003 004
EP 5-14	002 002	002 002	005 011	010 010	004 011	016 016	010 010	001 001	010 012	003 003
EP 5-15	001 002	002 002	016 016	007 007	004 012	005 016	019 020	004 004	011 011	013 013
EP 5-16	001 002	003 003	016 016	002 002	011 011	007 007	003 016	004 004	011 011	009 013
EP 5-17	002 002	002 003	011 011	002 004	004 011	005 015	005 005	001 004	011 011	004 005
EP 5-18	002 002	002 002	011 013	004 017	011 011	007 014	003 008	001 004	011 012	003 004
EP 5-19	002 002	003 003	003 003	011 011	011 011	002 005	003 012	003 004	011 011	009 009
EP 5-20	002 002	002 002	008 014	004 004	011 013	002 005	004 004	001 003	009 009	003 003
EP 5-21	002 002	002 002	008 013	004 017	011 013	015 016	003 017	003 004	011 011	003 003
EP 5-22	002 002	002 003	004 011	005 005	004 011	010 010	003 010	003 004	011 014	004 009
EP 5-23	002 002	002 004	011 016	004 007	011 012	003 006	003 019	004 004	011 011	004 004
EP 5-24	002 002	002 002	002 016	003 003	011 014	014 014	009 009	001 004	011 012	004 009
EP 5-25	002 002	002 002	006 013	003 003	237 011	005 014	010 010	001 004	006 006	004 004
EP 5-26	002 002	002 003	002 008	017 030	011 011	007 007	005 016	001 004	011 011	004 004
EP 5-27	002 002	002 002	008 008	002 004	011 015	016 016	009 009	003 004	011 011	007 009
EP 5-28	002 002	002 002	011 013	003 004	011 011	014 014	009 015	001 004	010 010	002 005
EP 5-29	002 002	002 003	011 016	010 024	005 011	002 002	014 019	004 004	011 011	005 005
EP 5-30	002 002	002 002	011 016	007 007	005 011	002 002	009 014	001 004	011 011	004 005
Failed to amplify	0	0	0	2	5	2	0	0	2	0

Results from MICRO-CHECKER analyses suggested null alleles were present at 6 out of 10 loci due to heterozygote deficiencies at these loci. The extremely low occurrence of failed amplifications (*i.e.*, homozygous for a null allele), however, suggests that deviations from HWE stemmed from other causes

Chapter 5

Western Atlantic introduction and persistence of

the marine bryozoan *Tricellaria inopinata*

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Western Atlantic introduction and persistence of the marine bryozoan *Tricellaria inopinata*

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Abstract

Most species of bryozoans have short-lived larvae with limited dispersal potential, yet many of these species possess global distributions. In this study, we report the first occurrence from the western Atlantic Ocean of the widely distributed arborescent bryozoan *Tricellaria inopinata* d'Hondt and Occhipinti-Ambrogi, 1985. This species was collected in Eel Pond, Woods Hole, Massachusetts, in September 2010. At that time, *T. inopinata* colonies had already formed dense conspecific aggregations at some collection sites, despite the presence of several other arborescent bryozoans. Sites were monitored throughout 2011 to track the success of this introduction, and to assess the reproductive timing of *T. inopinata* in Eel Pond. To determine the likelihood of *T. inopinata* persisting in Eel Pond and competing with previously established bryozoans, rates of metamorphic initiation, metamorphic completion, and overall offspring survivability were compared to one of the other dominant arborescent species. Finally, we provide taxonomic details to aid in identifying these animals, consider the potential mode of transport, and discuss the potential ecological implications resulting from this introduction.

Key words: biological invasions, Bryozoa, anthropogenic dispersal, fouling, non-indigenous species

Introduction

The unintentional transport of organisms *via* shipping traffic is a well-known means of dispersal for many marine species (e.g., Allen 1953; Carlton 1985; Carlton and Geller 1993). Indeed, anthropogenic transport has a disproportionate effect in certain phyla, allowing for numerous species to achieve distributions that far exceed their inherent dispersal potential. Such is the case for the phylum Bryozoa, which is dominated by sessile species that have short-lived larvae with limited dispersal capability. For instance, *Bugula stolonifera* Ryland, 1960 releases non-feeding larvae that will usually initiate metamorphosis within four hours of release (e.g., Woollacott et al. 1989; Wendt and Woollacott 1999). Due to anthropogenic dispersal, however, this species can be found in sub-tropical and temperate waters worldwide (see Rodgers and Woollacott 2006, Ryland et al. 2011). Watts et al. (1998) examined the geographic distribution of 197 globally distributed species of bryozoans and found that species abundance coupled with the animals' ability to foul, best explained the observed distributions. We report here on the introduction, establishment, and potential ecological implications of another widely distributed bryozoan, *Tricellaria inopinata* d'Hondt and Occhipinti-Ambrogi, 1985. Prior to our study, *T. inopinata* was not known to occur on the western side of the Atlantic Ocean. In 2010, however, colonies of this arborescent species were recovered in Eel Pond, Woods Hole, Massachusetts, where they have established a persistent population that is poised to spread to surrounding areas.

Tricellaria inopinata is a recently described species that was first found in a small portion of the Lagoon of Venice in 1982. Because of the ongoing long-term surveying effort within the lagoon stemming from 1978, it was thought to have been a recent introduction (d'Hondt and Occhipinti-Ambrogi 1985). Although the vector of transport that introduced these animals into the area was unknown, it has been hypothesized that the introduction could have occurred *via*

shipping traffic, or in association with the shellfish fishery (Occhipinti-Ambrogi 1991; 2000). By 1989, *T. inopinata* colonies could be found throughout much of the lagoon (area ≈ 550 km^2), and were seemingly only restricted by areas that routinely received an influx of fresh water (Occhipinti-Ambrogi 1991). *Tricellaria inopinata* spread throughout the lagoon despite the presence of numerous, previously established bryozoans and was found to overgrow several other species of arborescent bryozoans. Additionally, *T. inopinata* was epibiotic on various other organisms, including mussels, sponges, ascidians, and barnacles (Occhipinti-Ambrogi 1991), documenting a generalist larval settlement pattern. Such a pattern could provide these animals with a competitive advantage after being introduced to new areas, particularly when available substrate is a limited resource.

Globally, the distribution of *T. inopinata* is disjointed, with populations reported on the Pacific Coast of North America, Japan, Australia and New Zealand, in addition to those described in the Mediterranean (see Occhipinti-Ambrogi and d'Hondt 1994 and references therein) and northern European waters (De Blauwe 2009). Specimens collected in the Pacific were originally identified as *T. occidentalis* Trask, 1857, and some confusion existed as to whether or not *T. inopinata* was synonymous with *T. occidentalis* (e.g., Gordon and Mawatari 1992). Dyrynda et al. (2000), however, re-analyzed descriptions and specimens of *T. inopinata* and *T. occidentalis*, and documented that sufficient anatomical differences existed between them to allow for their identification as separate species. Further, these authors concluded that material collected from the Pacific that was anatomically similar to *T. inopinata* from the Adriatic and Atlantic should be assigned to *T. inopinata*. Shortly after its establishment and spread in the Venice Lagoon, *T. inopinata* was found in the Atlantic in 1996 in the northwest of Spain (Fernández-Pulpeiro 2001). The species was subsequently collected in southern England in 1998 (Dyrynda et al. 2000), in various locations in the Netherlands, Belgium, and France in 2000 (De Blauwe and Faasse 2001), and has recently been reported in Wales and Ireland (Ryland et al. 2009). Prior to our report, however, *T. inopinata* had not been reported elsewhere in the Atlantic. In this study, we document the first occurrence of *T. inopinata* in the western Atlantic Ocean, and provide taxonomic details to aid in identifying this species. Additionally, we provide insight into the reproductive timing of the populations established in Eel Pond, as well as empirical data on offspring survival and timing of metamorphic initiation and completion, in comparison to a dominant Eel Pond bryozoan, *B. stolonifera*.

Methods

As part of an ongoing research program, bryozoan assemblages in Eel Pond have been continuously monitored since 2006. *Tricellaria inopinata* was not known to occur in the area, but was found at several collecting sites in 2010. These sites were followed for the remainder of 2010, and throughout 2011, to track the success of this initial introduction, and to assess the survivability and reproductive timing of *T. inopinata* in Eel Pond. To aid in this, PLEXIGLAS® settling plates (15 × 15 cm) were submerged in early April 2011 under the Woods Hole Marine Biological Laboratory pier. At the time of submergence, none of the species of erect bryozoans that survived over winter in Eel Pond were found to possess polypides. Settling plates were routinely examined for bryozoan ancestrulae using a dissecting scope. To be able to continually monitor new recruitment, the settling plates were scraped clean after examination.

Induction of larval release

To assess reproductive effort over time, as well as to procure larvae for subsequent experimentation, bryozoan colonies were routinely collected and induced to release larvae. Bryozoans collected from Eel Pond were returned to the laboratory and maintained overnight in 38-liter glass aquaria equipped with a power filter providing water flow and aeration. Unfiltered seawater (UFSW) collected from Eel Pond concurrent with animal collection was used in the aquaria, and the temperature was set to mimic ambient water temperature at the time of collection. To induce larval release, dark-adapted colonies were removed from the aquaria, transferred to 1.5-liter glass bowls containing UFSW, and exposed to fluorescent light. Many bryozoan larvae are positively phototactic on release and will aggregate on the illuminated side of the bowl, facilitating larval collection. Groups of larvae were transferred to polystyrene weighing dishes, which were then placed in the dark to induce larval settlement. After the

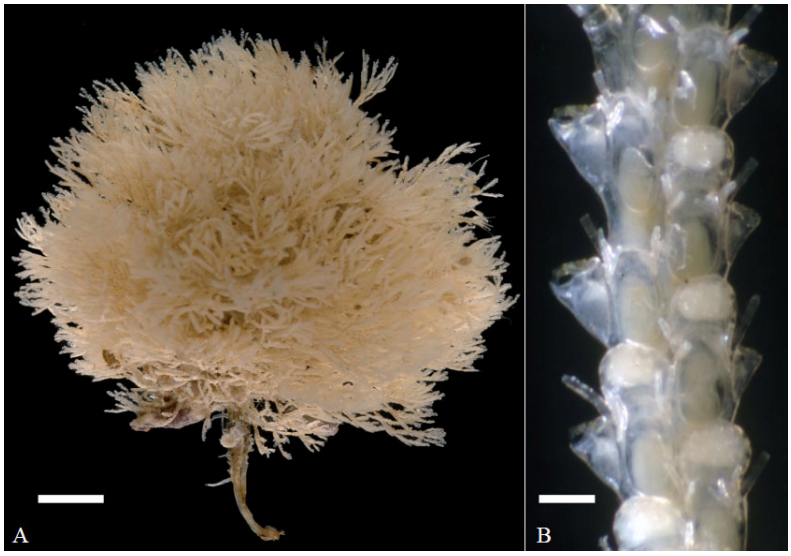

Figure 1. *Tricellaria inopinata* colony (**A**) and close-up of an individual branch (**B**) showing biserially arranged autozooids, large lateral avicularia, and filled ovicells. The specimen was fixed in 95% EtOH prior to imaging, causing the embryos to loose pigmentation and appear white. Scale bars = 5 mm (**A**) and 150 μm (**B**).

majority of individuals had initiated metamorphosis, the dishes were transferred into the aquaria and maintained there until completion of metamorphosis.

Offspring survival and timing to metamorphic initiation and completion

As a means to assess overall health of *T. inopinata* colonies in Eel Pond, as well as to determine the likelihood of this species establishing and competing with other bryozoans, experiments were conducted examining the time to metamorphic initiation, time to metamorphic completion, and overall offspring survivability, as compared to one of the other dominant arborescent bryozoans in Eel Pond, *B. stolonifera*. Gravid colonies of both species were collected on 27 July 2011 and maintained in the dark in glass aquaria. Larval release was conducted as previously described with one exception. Approximately 45 min after exposure to light, all larvae from both species were removed from the glass dishes and discarded. Larval release was allowed to occur for an additional 15 min, after which larvae were sampled and immediately utilized in the experiment. With this modification, we were able to ensure that larvae only differed in age by up to 15 min. Ten groups of larvae (*T. inopinata*: n=14-23; *B. stolonifera*: n=19-24) were transferred to polystyrene weighing dishes and placed in the dark, as light has previously been shown to effectively prevent metamorphic initiation in bryozoans under laboratory conditions (e.g., Wendt 1996). Initiation of metamorphosis was assessed hourly for a total of four hours, after which free-swimming larvae were counted, removed, and the dishes then submerged in glass aquaria. Metamorphic completion was initially assessed at 18h after release, and then in two-hour intervals until both species had achieved a 95% completion rate. The experiment was allowed to continue for a total of 72h, at which time those individuals that had not completed metamorphosis were counted and the experiment terminated.

Results

Taxonomic description

Colonies of *Tricellaria inopinata* from Eel Pond appear whitish-grey to straw-colored and grow as erect, compact tufts (Figure 1), generally not exceeding 4 cm in height. Superficially, colonies resemble two other common Eel Pond bryozoans, *Bugula stolonifera* and *B. simplex* Hincks, 1886; due to heavier calcification in *T. inopinata*, however, the species can be distinguished by touch. Additionally, anatomical differences become readily apparent under even slight

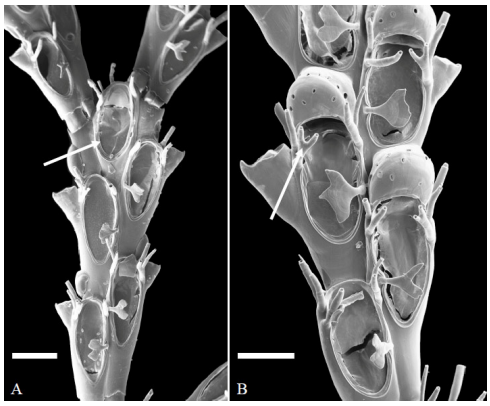

Figure 2. SEM of non-ovicellate (**A**) and ovicellate (**B**) *Tricellaria inopinata* autozooids. The scutum, a modified spine that partially covers the frontal membrane, is prominent and highly variable, ranging in shape from slender to broad and from forked to wavy. Occasionally, it is missing entirely (**A**). Autozooid spines are prominent as well, and the most basal of the 3 external spines is often forked (**B**). Scale bars = 200 µm (**A**) and 150 µm (**B**).

magnification, and the following characteristics can be used to distinguish *T. inopinata* from other erect bryozoans. In colonies of *T. inopinata*, zooids are arranged bi-serially and do not possess vibracula (Figure 2). Moveable pedunculate avicularia, similar to those described in *Bugula* spp., are not found in *T. inopinata*. Large lateral avicularia, however, are found on many, but not all, zooids. Pronounced spination about the operculum of the zooid is common in this species, with generally two internal and three external spines for each zooid. The most basal of the three external spines is often forked, but this characteristic is not constant within colonies. The scutum is prominent in this species, but the shape can vary dramatically within an individual colony from slender to broad and from forked to wavy (Figure 2). In some instances, the scutum was missing entirely from zooids immediately preceding a bifurcation (Figure 2a). Ovicells are situated distally to the maternal zooid and are multi-pored. The height and width of the ovicells was approximately equal (n = 20), a consistent characteristic within and among colonies.

Larvae of *T. inopinata* have previously been described in detail (Occhipinti-Ambrogi and d'Hondt 1994). They are barrel-shaped non-feeding coronate larvae, also referred to as buguliform. Expanded coronas extend aborally, equatorially, and orally in position, with small pallial sinuses (type AEO/ps) (see Zimmer and Woollacott 1977). Early-stage embryos can appear pink while in the ovicell, but larvae are cream-colored with orange-red eyespots. As with the larvae of many bryozoans that brood their embryos, *T. inopinata* larvae are positively phototactic on release and rapidly initiate metamorphosis once sequestered in the dark. Completion of metamorphosis results in a squat ovoid ancestrula that lacks a scutum (Figure 3). Ancestrular spines are pronounced, although spine length is highly variable. Spines generally number between 8 and 10, and can be arranged symmetrically or asymmetrically around the operculum. Characteristic of most of these newly metamorphosed individuals, are two rhizoids that often proceed down the length of the ancestrula and expand into broad to tripartite tips (Figure 3).

Observation of occurrence in Eel Pond

Tricellaria inopinata colonies were first collected from Eel Pond in September 2010 (Salinity = 34 psu, Temperature ≈ 25ºC). During a routine collection conducted in July, these animals were not observed. Rather, collection sites were dominated by two arborescent bryozoans common to the area, *B. stolonifera* and *B. turrita* Desor, 1848. Prior to the September collection, however, there was a dieback of both of these species, possibly due to decreased salinity in Eel Pond resulting from heavy rainfall in late August and early September (http://water.weather.gov/precip/). At the time of first observation, *T. inopinata* could be found attached to submerged substrates throughout Eel Pond. Indeed, because of the decrease in abundance of the two previously dominant bryozoans, *T. inopinata* had already begun to form dense aggregations at several sites. Additionally, *T. inopinata* colonies were found to be epibiotic on several different Eel Pond organisms, including fucoid algae and the solitary ascidian *Styela clava* Herdman, 1881 (Figure 4), as well as on surviving *B. stolonifera* and *B. turrita* colonies. Aggregations of *T. inopinata* persisted throughout the fall, but began to diminish in early December (34 psu, 5ºC). By January 2011 (35 psu, 4ºC), *T. inopinata* colonies had died back, leaving only a few, sporadic isolated colonies. Some of these colonies survived the near-freezing temperatures and ice formation common to Eel Pond in the

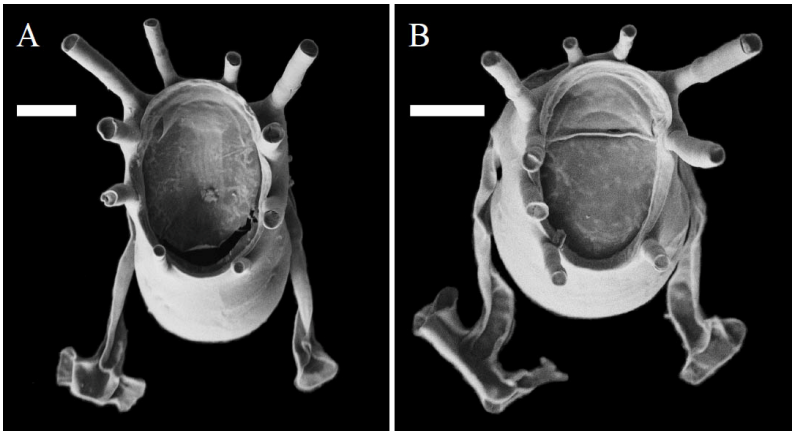

Figure 3. SEM of *Tricellaria inopinata* ancestrulae. Ancestrulae possess conspicuous spines surrounding the operculum, which generally number between 8 and 10. All ancestrulae lack a scutum, and most possess two rhizoids that aid in attachment. Scale bars = 75 µm (**A**) and 50 µm (**B**).

Figure 4. *Tricellaria inopinata* colonies growing on the solitary ascidian *Styela clava* (**A**) and the fucoid alga *Ascophyllum nodosum* (**B**). Scale bars = 1 cm.

winter and persisted through March 2011 (36 psu, 5ºC), but no functional autozooids were found in any collected colony through this time.

Reproductive timing of Tricellaria inopinata *in Eel Pond*

Collection sites within Eel Pond were monitored weekly beginning in March 2011 for initial colony re-growth. No sign of re-growth was observed until late May (35 psu, 14ºC). Colonies that overwintered were found to possess newly budded autozooids at the tips of the colonies, although no functional autozooids were found in the interior of the colony. Additionally during this time period, numerous small colonies were observed, potentially having arisen from over-wintering rhizoids. Functional autozooids were found throughout these smaller colonies. None of the autozooids on any collected colony possessed a filled ovicell, nor were any ancestrulae found on submerged settling plates. By early June (35 psu, 17ºC), colonies were found to possess brooded embryos, which appeared pink in the multi-pored ovicells. None of the collected colonies were found to release larvae after exposure to light. Within one week, however, collected colonies were found to release larvae, and numerous *T. inopinata* ancestrulae and juveniles were found growing on the submerged plates (33 psu, 20ºC). By late June (33 psu, 21ºC), collected colonies possessed numerous brooded embryos within the colony, and exposure to light resulted in the release of thousands of larvae from collected colonies. High rates of larval release were found throughout the summer and fall (31-35 psu, ≤ 25ºC), but began to decrease in mid-December (35 psu, 8ºC). Reduced larval output was observed in collected colonies until early January (35 psu, 6ºC), when approximately 35 colonies released only 8 larvae. None of these larvae initiated metamorphosis, and no brooded embryos were found in any colony examined after release.

Offspring survival and timing to metamorphic initiation and completion

Both species tested experienced high rates of metamorphic initiation and completion over the duration of the experiment (Figure 5). For *B. stolonifera*, 213 out of 216 (98.6%) of the larvae sampled initiated metamorphosis, and 210 (98.6 %) of those that initiated completed metamorphosis. For *T. inopinata*, 174 out of 185 (94.1%) larvae initiated metamorphosis, of which 169 (97.1%) completed metamorphosis. Timing for metamorphic initiation and completion were similar for the two species as well. For *B. stolonifera*, 90% of sampled individuals initiated metamorphosis within 1h, while 90% completed metamorphosis within 30h after release (Figure 5). For *T. inopinata*, 90% of sampled individuals initiated metamorphosis within 2h, and 90% had completed metamorphosis within 32h.

Discussion

Taxonomic verification

As documented in previous descriptions of *Tricellaria inopinata* (e.g., d'Hondt and Occhipinti-Ambrogi 1985; Dyrynda et al. 2000; De Blauwe and Faasse 2001), colonies collected in Eel Pond displayed a high degree of anatomical variation (see Figures 2 and 3). For instance, spine count, pattern, and size were found to vary across ancestrulae. In adults, the presence of a bifid spine was inconsistent from zooid to zooid, as was the presence of lateral avicularia. Perhaps the most striking example, however, occurred in the shape and size of the scutum, which displayed large amounts of variation even within a colony. Indeed, it was this type of anatomical variation that initially led to confusion as to the proper identification of these animals, relative to previous species' descriptions of other *Tricellaria* congeners. In their description of the bryozoans of New Zealand, Gordon and Mawatari (1992) remarked that it was puzzling that *T. inopinata* was erected as a new species, as the description given by d'Hondt and Occhipinti-Ambrogi (1985) was within the range of variation for *T. occidentalis*. As previously mentioned, however, Dyrynda et al. (2000) re-analyzed descriptions and specimens of *T. inopinata* and *T. occidentalis*, and concluded that the scutum was one of the distinguishing features for these species, and described the scuta in *T. occidentalis* as "invariably slender or only slightly spatulate." Further, according to d'Hondt and Occhipinti-Ambrogi (1985) and De Blauwe and Faasse (2001), only two other *Tricellaria* species possess multi-pored ovicells: *T. occidentalis* and *T. prasescuta* Osburn, 1950. In *T. occidentalis*, the ovicell is reported to be 1.5-2.0 times wider than it is high, in *T. prasescuta* the ovicell is reported to be 1.5-2.0 times higher than it is

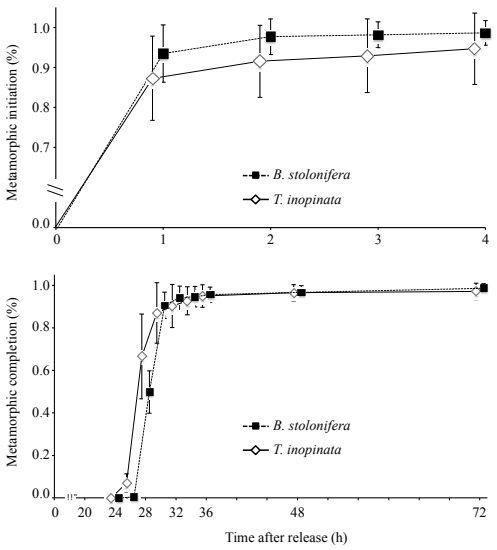

Figure 5. Percentage of individuals initiating and completing metamorphosis over time for *Bugula stolonifera* and *Tricellaria inopinata* offspring. Similar rates of overall survival, metamorphic initiation and metamorphic completion were observed between the two species throughout the duration of the experiment. Bars = 1 S.E.

wide, while in *T. inopinata* the ovicell height and width are roughly equal. Hence, colonies collected in Eel Pond are characteristic of *T. inopinata*.

Vector of transport

As with the invasion by *T. inopinata* into the Mediterranean, it remains unclear how these animals were transported across the Atlantic Ocean and introduced to the Woods Hole, MA region. Occhipinti-Ambrogi (1991, 2000) suggested shipping traffic and the shellfish fishery as likely vectors that introduced *T. inopinata* to the Mediterranean. Due to a lack of an appropriate aquaculture fishery in the Woods Hole region, it is unlikely that *T. inopinata* could have been introduced in such a manner. Therefore, shipping traffic appears to be the most likely vector. As previously stated, shipping has been implicated in the dispersal of many marine organisms. For instance, Schwaninger (1999) provided convincing genetic evidence that the invading population of the bryozoan *Membranipora membranacea* Linnaeus, 1767 in the Gulf of Maine stemmed from populations in northern Europe. For the introduction of *T. inopinata*, there are no major shipping lanes that include the Woods Hole region, but there are vessels that routinely conduct trans-Atlantic voyages that could potentially connect Woods Hole to northern Europe or the Mediterranean. The Woods Hole Oceanographic Institution possesses several ships capable of trans-Atlantic voyages. For example, in 2008 the Research Vessel (R/V) *Knorr* travelled from Woods Hole to northern Europe and back in late summer and fall (http://strs.unols.org/Public/diu_schedule_view.aspx?ship_id=10037&year=2008). More recently, the R/V *Knorr* travelled to Aveiro, Portugal, in July 2010, and returned to Woods Hole on August 1, 2010 (http://strs.unols.org/Public/diu_schedule_view.aspx?ship_id=10037&year=2010). Interestingly, *T. inopinata* was reported in a nearby, heavily used port in Ria de Aveiro (Marchini et al. 2007). While it seems unlikely that an erect bryozoan colony attached to a ship's hull could survive the trans-Atlantic voyage, it is worth noting that many arborescent bryozoans undergo an annual cycle of colony die-back and re-growth. During this cycle, the arborescent portion of the colony will die off, most likely due to deterioration in environmental conditions. When conditions improve, however, colonies will grow back, presumably stemming from the root-like projections that remained attached to the substrate. Numakunai (1967) found that *B. neritina* Linnaeus, 1758 rhizoids collected during winter budded zooids after approximately 10 days of incubation at 20ºC. Hence, if even a portion of the rhizoids survived the trans-Atlantic trip, it remains possible that at the completion of the voyage, a new zooid could form that would eventually develop into a reproductively mature colony.

Ecological implications

Shortly after its initial description in the Venice Lagoon, *T. inopinata* was documented to undergo a rapid range expansion, colonizing most of the lagoon and spreading to various localities in the northeastern Atlantic (e.g., Occhipinti-Ambrogi 1991; De Blauwe and Faasse 2001). Further, this species not only spread rapidly, but also appears to have had a negative effect on previously established bryozoan populations. For instance, *T. inopinata*

in Venice Lagoon was initially observed to co-exist with several bryozoan species that possessed similar growth forms (Occhipinti-Ambrogi 1991). Shortly thereafter, however, the previously established bryozoan populations decreased in abundance, such that *T. inopinata* became the dominant species at these collection sites (see Occhipinti-Ambrogi 2000). A similar phenomenon could be occurring in Eel Pond. Prior to 2010, the dominant bryozoans in Eel Pond for the majority of the reproductive season were *Bugula stolonifera* and *B. turrita*. Indeed, *B. stolonifera* was commonly found forming dense aggregations on much of the available substrate, essentially carpeting floating docks and pier pilings where it occurred. After the observed introduction of *T. inopinata* in 2010, all three species were found to become abundant late in the reproductive season. Throughout 2011, however, *B. stolonifera* never reached the abundance that had been observed in previous years, and by mid-season was completely absent from several collecting sites, which were dominated by *T. inopinata*. *Bugula turrita* was also found in reduced abundance, although its decline was not as drastic. It is unclear why this decrease in abundance occurred, but it could be a consequence of reproductive timing and competitive advantage by *T. inopinata*.

Although the timing to metamorphic initiation and completion and overall survival between *B. stolonifera* and *T. inopinata* were similar (Figure 5), there were differences in onset of reproduction in the two species. In 2011, the onset of reproduction in *T. inopinata* occurred in early June, and by mid-June, numerous ancestrulae and young colonies were found on the submerged settling plates. In contrast, the onset of reproduction in *B. stolonifera* did not occur until late June. This difference in timing could have provided *T. inopinata* sufficient time to recruit to available substrate and begin growing, preventing *B. stolonifera* from forming dense aggregations where it had done so previously. Alternatively, the ability of *T. inopinata* to overgrow local species, as has been previously documented, could be the overriding factor. Throughout the summer in Eel Pond, numerous *T. inopinata* ancestrulae and young colonies were found attached to *B. stolonifera* and *B. turrita* colonies. Conversely, very few *T. inopinata* colonies were observed with non-conspecific individuals attached. Conspecific larval settlement, whereby larvae attach and metamorphose on adults of the same species, can be common in some bryozoans (e.g., Johnson and Woollacott 2010). The ability of *T. inopinata* larvae to foul and grow on other bryozoan species, coupled with the inability of other species to settle on *T. inopinata* adults, could provide a competitive advantage that allows this species to outcompete previously established arborescent bryozoans, even after a recent introduction. It remains unknown what effect this type of settlement has on growth and reproductive output of the previously established bryozoans. What appears clear, however, is that within a year of its first observance in Eel Pond, *T. inopinata* has established itself as the dominant bryozoan despite the presence of several previously established arborescent species, and appears poised to spread to surrounding areas. The species' rapid range expansion and increase in the northeastern Atlantic since its introduction to European and British shores in the 1990s, particularly its recent success in southern England (Arenas et al. 2006), highlights the need for periodic monitoring of nearby coastal areas.

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Chapter 6

Conclusions

The potential advantages of self-fertilization to simultaneous hermaphrodites are numerous. Sexual reproduction requires the fusion of gametes, with each gamete containing 50% of its parent's genome. This reduction in parental legacy has been termed the cost of meiosis (Williams 1975) and can be avoided by selfing. Self-fertilization can also alleviate some of the risks associated with sex, including finding an appropriate mate or, for spawning species, dilution of gametes. Additionally, the ability to self-fertilize could allow some species to rapidly colonize and spread in a new area, regardless of the presence of conspecifics. Long-distance dispersal events can lead to the introduction of small numbers of individuals into a new area, but theoretically a single self-compatible individual could colonize an area following this type of introduction (Baker 1955). This latter advantage could help explain why many species of bryozoans with short-lived larvae have obtained global distributions.

The marine bryozoan *Bugula stolonifera* Ryland, 1960 broods embryos within the colony, and releases short-lived non-feeding larvae that will usually commence metamorphosis within 1-4h after release (Woollacott et al. 1989). Despite this short dispersal stage, these animals have can be found in temperate and sub-tropical waters worldwide (Rodgers and Woollacott 2006). While this pattern is likely primarily due to anthropogenic dispersal, this documented distribution leaves no doubt as to the ability of these animals to colonize a new area after a long-distance dispersal event. Results from my dissertation suggest that this ability is not due to self-fertilization. In Chapter 2, I show that selfing in *B. stolonifera* results in the release of viable offspring, but these individuals experience a significant reduction in fitness. As compared to outcrossed controls, this is expressed as significantly fewer larvae released, reduced rates of

metamorphic initiation and completion, and decreased survival and fecundity. Indeed, no viable larvae were collected from reproductively mature selfed colonies. These results suggest that although capable of selfing, it is not routinely occurring in natural populations of this species. How these animals minimize selfing is not clear, but it could be a consequence of how *B. stolonifera* grows naturally. Many arborescent bryozoans, including *B. stolonifera*, are commonly found growing in dense conspecific aggregations. Having high numbers of individuals on small spatial scales could simply minimize the likelihood that a colony utilizes its own sperm for fertilization. Further, rather than possessing the ability to self-fertilize, it could be that these aggregations are also responsible for making these animals such successful colonizers.

Inherent in many long-distance dispersal events is a reduction in genetic diversity associated with the introduced population, relative to the source population. Hamner et al. (2007) documented that genetic diversity for populations of two species of lionfish introduced to the western Atlantic was lower than for populations found in these species' native range. Dupont et al. (2007) investigated the genetic diversity of introduced populations of the ascidian *Corella eumyota* Traustedt, 1882 and found that newly established populations had lower diversity than putatively native populations. Interestingly, despite the reduced genetic diversity associated with these introductions, both the ascidians and lionfish were able to colonize, spread, and thrive in their introduced areas. These results document that an inherent resilience to inbreeding could allow a species to overcome the founder effect. Alternatively, multiple invasions by a species to an area could also lead to increased colonization ability. Roman (2006) investigated the invasion of the green crab *Carcinus maenas* (Linnaeus, 1758) to New

England. These animals were first introduced to the U.S. in 1817, where they were found in New York and southern Massachusetts. Although it progressed northward, the invasion stalled on the Scotian Shelf, until a sudden range expansion northward occurred in the 1980s. Originally thought to coincide with warming waters, Roman (2006) used mitochondrial DNA to document that the sudden expansion coincided with increased genetic variability in the most northern population. He concluded that multiple cryptic introductions, most likely from the northern end of the crab's native range in Europe, allowed for the spread into waters that were too cold for the original southern invasion front. Therefore, repeated introduction of new alleles can also allow for an introduced species to overcome a potential founder effect. As previously discussed, *B. stolonifera* is not resilient to inbreeding, and it seems unlikely that multiple cryptic invasions are responsible for this animal's global distribution. That these animals are such successful colonizers would appear paradoxical, but results from fine-scale population genetic analyses in *B. stolonifera* provided a potential solution to this paradox.

In Chapter 3, I investigated the genetic make-up of conspecific aggregations of *B. stolonifera*, and documented high larval mixing within these aggregations. A group composed of a colony and its attached individuals (area ≤ 21 cm^2) had as much genetic variability as was found for the entire sampling site (area ≈ 15 m^2). Parentage exclusion analyses documented that the majority of attached individuals ($> 93\%$) could not have originated from the colony on which they were found. Finally, the majority of attached individuals within a group ($\approx 63\%$) shared less than a half-sibling relationship with one another. Taken together, these results suggest that any low-probability long-distance dispersal events that might only transport a few individuals across entire ocean basins

could introduce as much genetic variability as was found in the source population. This increased genetic diversity could allow these animals to avoid the founder effect altogether, enabling them to rapidly colonize and spread following a single introduction event. Just such an introduction was observed in Eel Pond for another bryozoan with a similar settlement and growth pattern.

In Chapter 5, I documented the first occurrence of *Tricellaria inopinata* d'Hondt and Occhipinti-Ambrogi, 1985 to the western Atlantic Ocean. Originally described from specimens collected in the Lagoon of Venice, *T. inopinata* is known to occur in various locales in the Pacific Ocean, as well as in the eastern Atlantic in northern European waters (see Occhipinti-Ambrogi and d'Hondt 1994; De Blauwe 2009). Prior to our report, however, *T. inopinata* had not been reported elsewhere in the Atlantic. Colonies of *T. inopinata* were first collected from Eel Pond in September 2010; these animals were not observed during a routine collection conducted in July. At the time of their collection, colonies had already begun to form dense conspecific aggregations at several sites around Eel Pond. Further, similar to *B. stolonifera*, numerous *T. inopinata* ancestrulae were found on conspecific adults. This introduction was followed throughout 2011, where *T. inopinata* was observed to possess high rates of growth and reproductive output. Although there are several other previously established arborescent bryozoans in Eel Pond, *T. inopinata* has established itself as the dominant bryozoan in the short time since its arrival. It has become apparent that, despite the trans-Atlantic dispersal, these animals do not appear to be suffering from a potential founder effect. To date, genetic analyses have not been performed for *T. inopinata* aggregations. Due to their similarity in conspecific settlement and aggregation formation, however, it seems likely that the fine-

scale population genetic patterns observed for *B. stolonifera* would also apply to *T. inopinata*. The high genetic variability on small spatial scales would allow these animals to avoid any potential founder effect following their long distance dispersal, enabling them to thrive in their new area after only a single introduction event. Further, this genetic variability could be the primary reason why many other species of bryozoans with limited dispersal capabilities are globally distributed, and are such successful invaders.

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